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CONGENITAL MALFORMATIONS INDUCED IN RATS BY MATERNAL VITAMIN A DEFICIENCY

II. EFFECT OF VARYING THE PREPARATORY DIET UPON THE YIELD OF ABNORMAL YOUNG [1]

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FOUR FIGURES

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Congenital anomalies in the offspring of vitamin A deficient animals have been described repeatedly. The literature on this subject has been reviewed by Warkany and Schraffenberger ('46). The studies of Hale ('33, '35, '37) are of great importance, since genetic factors could be ruled out as the cause of the malformations obtained. Hale fed gilts of known stocks a diet deficient in vitamin A and possibly in other nutritional factors. The offspring showed anophthalmos or microphthalmos, accessory ears, harelip and cleft palate, subcutaneous cysts and misplaced kidneys. For experimentation on a large scale the production of congenital malformations by maternal vitamin A deficiency in a small laboratory animal appeared desirable. Anderson ('41) reported briefly the occurrence of diaphragmatic hernia in about 25% of the young of rats bred on a diet containing the minimum amount of vitamin A required for pregnancy and parturition. A small number of the controls which were fed halibut liver oil, showed the same lesion.

[1] This work was aided in part by a grant from the Nutrition Foundation, Inc., New York.

Warkany and Schraffenberger ('44, '46) reported congenital anomalies of the eyes in the offspring of rats that had been depleted of vitamin A. Female rats were raised on a preparatory diet (diet U) which contained small measured amounts of carotene. The carotene supplement was necessary to make possible growth and maturation of the females; however, it was insufficient to provide vitamin A for storage. When the female rats reached a weight of 150 to 160 gm and had regular oestrous cycles they were given a purified diet completely free of vitamin A and carotene (diet W). While on this diet the females were bred and kept throughout pregnancy. Under such dietary conditions few animals carry their young to term. Many females become sterile; some stop having oestrous cycles; others mate but resorb their young. In 1 series of experiments (Warkany and Schraffenberger, '46) only 7 of 140 females carried their offspring to term and 39 young were obtained. All the eyes of the young that were serially sectioned were found to be abnormal. The most constant abnormal finding was a fibrous retrolenticular membrane in place of the vitreous body (fig. 1B-H). In addition there were frequently colobomas, eversion, abnormal structure and folding of the retina, rudimentary development of the iris and of the ocular chambers, defects of the cornea and of the conjunctival sac, and lack of fusion of the lids. In the last case the abnormal eyes could be recognized by external inspection ("open eyes") (fig. 2). These experiments on rats were repeated with slight modifications by Jackson and Kinsey ('46) who confirmed the findings of Warkany and Schraffenberger. The study of Jackson and Kinsey was supplemented by determinations of the vitamin A level of pregnant rats which was found to be less than 12 I.U. per 100 ml in all the mothers who produced young with abnormal eyes. In the series of these authors only 4 of 57 females subjected to the dietary conditions described carried their fetuses to advanced stages of development, and Jackson and Kinsey concluded "that the ocular defects occur in the young rat only when the maternal vitamin A deficiency is extremely severe, so ad-

vanced, in fact, that fetal resorption is common and normal birth is impossible.''

Since the rate of fertility was very low in the original experiments and the yield of abnormal offspring very small, improvements of the experimental methods were attempted by modifications of the preparatory diet. These modifications are reported in the present communication. In the previous experiments rats of the Sprague-Dawley strain only were used. It seemed advisable to repeat the experiments with rats from a different source and in this series a large number of rats obtained from the Albino Farms, Red Bank, N. J. were included.

METHODS

Female rats weighing 30 to 40 gm were placed on the preparatory diet (diet U) which had the following percentage composition: Ground whole wheat, 74; crude casein,[2] 15; brewers' yeast,[3] 10; sodium chloride of C.P. grade, 1. According to the supplements added to this diet the animals were divided into 4 groups. One hundred and sixty-five females were given a supplement of only 4 µg of carotene every tenth day (group I). Since this supplement proved insufficient for growth and maturation, each rat received in addition approximately 2 gm of frozen horsemeat daily. This group was kept on diet U until mating occurred, at which time the females weighed between 150 and 160 gm. Then the females were placed on diet W. Group II, consisting of 34 females, received a supplement of 12 µg carotene every tenth day. In group III 33 animals were given 25 µg carotene every tenth day. A fourth group consisting of 28 females received during the first month supplements of 25 µg of carotene every tenth day. In the following month a loss of weight was observed and therefore the carotene intake was doubled during the last 2½ weeks of the preparatory period. Groups II, III and IV were placed on diet W upon reaching maturity and attempts to breed them were made after the change to this purified diet. In these 3 groups mating took place usually within 1 week or 10 days after the

[2] Borden. [3] Mead Johnson.

change to diet W; but even after 4 weeks on the purified diet mating occasionally occurred. Diet W, a purified diet completely free of vitamin A and carotene, had the following percentage composition: Sucrose, 68; vitamin test casein, 18; vegetable oil, 10; salt mixture of Hubbell, Mendel and Wakeman ('37), 4. To 100 gm of this diet was added 0.8 μg thiamine hydrochloride; 0.8 μg pyridoxine hydrochloride; 0.8 μg riboflavin; 1 μg calcium pantothenate; 10 μg niacinamide; and 100 μg choline chloride. This diet was supplemented every tenth day by vitamin D (1 drop of a 1:4 dilution of drisdol with olive oil), vitamin E (1 drop of a solution of 5 gm alphatocopherol in 100 ml of olive oil), and vitamin K (1 drop of a solution of 1 μg of 2-methylnaphthoquinone in 5 ml of olive oil). On this diet the female rats were bred and kept throughout pregnancy. Vaginal smears were made daily and the weight curves were closely observed. This made possible the recognition of the phase of oestrus; the beginning of pregnancy (by finding sperm); the continuation of pregnancy; or beginning of resorption (by finding of large amounts of blood in the vaginal smear and by a marked loss of weight). The day on which sperm were found was counted as the first day of pregnancy. The profuse bleeding of resorption can be distinguished as a rule from the normal blood sign, which appears between the tenth and the fifteenth days of gestation and which consists of a small amount of blood in the vaginal smear. When early termination of pregnancy threatened, the animal was opened and the fetuses removed. When the young were carried to term the mothers were opened on the twenty-second day of gestation. Many young thus removed were alive but none could be raised. The mothers were sacrificed and therefore no second litters were obtained. The eyes of at least 1 animal in each litter were sectioned serially. If no retrolenticular membrane was found the animal was considered normal but if a membrane was seen in any section the animal was considered abnormal. When the eyes of 1 young in a litter were found to be abnormal the entire litter was considered and counted as abnormal. This method of classification appeared

to be justified, since in 14 litters selected at random the eyes of all the littermates when serially sectioned were found to be so similar that all the members of each litter could be considered normal or abnormal (fig. 1). There was also marked external resemblance between the animals of the same litters (fig. 2).

RESULTS

Of 260 females reared on the preparatory diet U, 22 had cycles but did not mate. One hundred and twenty-four females mated but did not have any issue. Litters were obtained from 114 females but only 30 of these litters were carried to term (table 1). The rest were obtained prematurely and consisted of fetuses of various gestational ages (table 2). Of the females used in this series 156 were of the Sprague-Dawley strain and 104 were obtained from the Albino Farms.

According to the presence or absence of a retrolenticular membrane in the eyes of 1 or of several members of a litter, 89 litters were considered abnormal and 25 normal. These litters consisted of a total of 820 young, 612 of which were considered abnormal and 208 normal. In addition to the retrolenticular membrane, other ocular defects resembling those described in our previous publication ('46) were seen in many of the eyes sectioned. In most eyes with a retrolenticular membrane the vitreous humour is absent and fibrous tissue occupies the entire space between the lens and the retina. However, in some of the abnormal eyes rudiments of the vitreous body are present and are transversed by a thick strand of fibrous tissue which starts at the head of the optic nerve and continues to the posterior surface of the lens (fig. 1). Many litters show external changes which are not observed in control animals. In figure 2 an abnormal litter of 7 young is compared to a normal newborn animal. The abnormals are smaller than the normal animal although they were removed from the mother's uterus on the twenty-second day of gestation. Owing to the edematous swelling of the subcutaneous tissue the narrowing at the neck is markedly reduced and the entire back presents a single convexity. All the animals of

this litter have "open eyes" which are best seen in specimens B and D. Subcutaneous hemorrhages are seen in C and D. The fingers and toes are well-developed but the dorsa of the

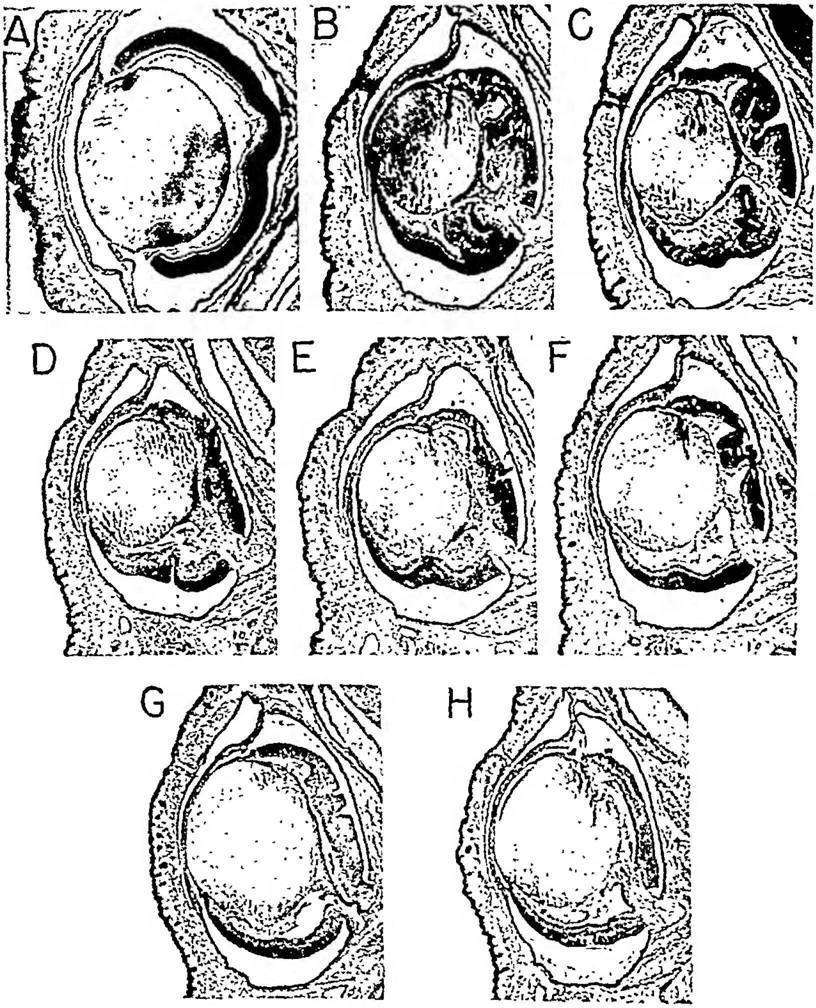

Fig. 1 Sections through eyes of newborn rats. A, control; B-H, offspring of vitamin A deficient mother. These sections represent the eyes of 7 littermates. The retrolenticular membrane is seen in every section, while only rudiments of a corpus vitreum are developed. A slight eversion of the retina is seen in section G only. (× 18).

hands and feet are puffy. The soles of the feet are turned inward which represents a retention of the fetal position.

In many young with abnormal eyes serial sections of the trunk and abdomen revealed various congenital anomalies of

Fig. 2 A, newborn rat, control; B-H, offspring of vitamin A deficient mother, removed from the uterus on the twenty second day of gestation. These young, all littermates, show edema and inward rotation of the feet. The "open eye" can be seen in B and D, a cutaneous hemorrhage in D.

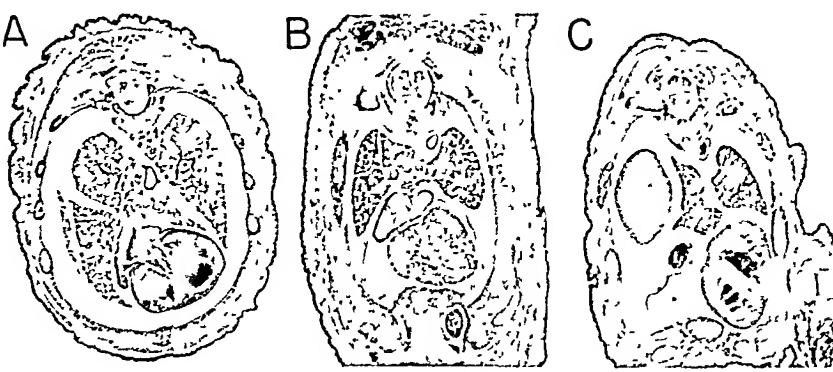

Fig. 3 Sections through the chest of newborn rats. A, control (× 3½); B and C (× 3½), offspring of vitamin A deficient mothers. In B the lung, the pleura and the heart muscle show retarded development. In C a lobe of the liver is seen in the right pleural cavity (diaphragmatic hernia).

the soft tissues. The lungs are underdeveloped and frequently retain their fetal position behind the heart and fail to grow forward. The pleural chambers border the pericardial cavity posteriorly and laterally but do not surround it (fig. 3 B and C). The heart muscle retains the spongy structure of the fetal

heart (fig. 3 B and C). In a number of specimens the right dorsolateral portion of the diaphragm is not developed and a lobe of the liver may protrude into the pleural space. This represents a diaphragmatic hernia (fig. 3 C). The kidneys are hypoplastic and the renal pelvis and the ureters are not distended as in the normal rat at the end of the gestational period. The space between the kidneys is narrow and fusion may take place in the lower poles, thus forming a horseshoe kidney. The testes are frequently undescended and may be found at the level of the kidneys or above (fig. 4 C). A detailed study

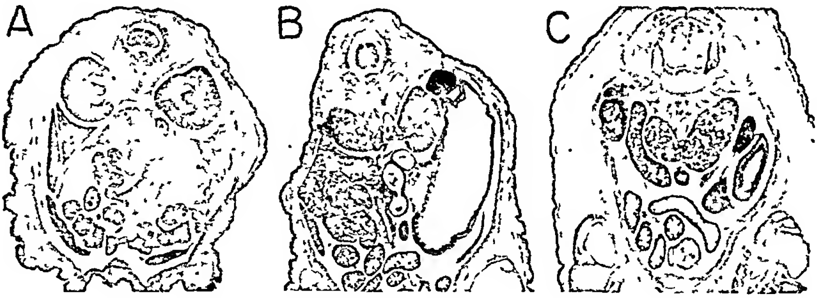

Fig. 4 Sections through the abdomen. A, control ($\times 3\frac{1}{2}$); B and C, offspring of vitamin A deficient mothers. In B the renal pelvis seen in the left kidney is not unfolded ($\times 5$). In C (20 day fetus; $\times 7$) a fusion of the lower poles of the kidneys is seen (horseshoe kidney).

of these anomalies is being made at the present time and will be reported elsewhere. There is a tendency for hemorrhages to occur in the skin, the eyes and other organs. Keratinizing metaplasia was found by Wilson and Warkany ('47) in many abnormal young in the urethra distal to the termination of the genital ducts. In other parts of the body no abnormal keratinization was observed.

In all 4 groups abnormalities of the same type were obtained and there was no difference between the abnormalities in the offspring of rats of the Sprague-Dawley strain and those obtained from the Albino Farms. However, there were differences in the incidence of abnormalities and in the fer-

tility rates in the 4 groups according to the different amounts of carotene in the preparatory period.

Groups I and II had the lowest fertility rate and the highest incidence of abnormal young; group IV had the highest fertility rate and the lowest incidence of abnormal young.

Table 2 shows that most of the younger fetuses that were dying in utero or were threatened by intrauterine death were

TABLE 1

Breeding results with females raised on the preparatory diet U with various supplements and bred on diet W.

CATEGORY OF INTEREST	TOTAL	SUPPLEMENTS OF PREPARATORY DIET			
		Group I 4 μg carotene[1] (and horsemeat)	Group II 12 μg carotene[1]	Group III 25 μg carotene[1]	Group IV 25–50 μg carotene[1]
Number of females	260	165	34	33	28
Cycles but no matings	22	17	3	2	0
Matings but no issue	124	88	19	13	4
Number of litters	114	60	12	18	24
abnormal	89	52	12	12	13
normal	25	8	0	6	11
Number of young	820	428	76	139	177
abnormal[2]	612	363	76	96	77
normal[2]	208	65	0	43	100

[1] The carotene supplements were given every tenth day. The animals in group I received 2 gm frozen horsemeat daily.

[2] These figures are based on the assumption that the entire litter was abnormal, when anomalies were found in the eyes of at least 1 member of the litter (see page 4).

TABLE 2

Gestational age of 114 litters and of 820 young.

DAY OF PREGNANCY	15th	16th	17th	18th	19th	20th	21st	22nd
Total number of litters	3	6	8	7	10	15	35	30
abnormal	3	6	7	7	9	15	25	17
normal	0	0	1	0	1	0	10	13
Total number young	14	28	47	24	79	105	272	251

abnormal, while about one-third of the survivors of the gestation period were normal.

Eighty females received in the same shipments as the experimental animals were used as controls. They were fed an adequate stock diet throughout their growing and breeding periods and mated to the same males as the females on the deficient diets. They delivered a total of 104 litters and 822 young. The eyes of 60 of the young, each representing a different litter, were serially sectioned. No retrolental membrane was found in any of the eyes examined. Sixty control offspring were dissected and neither diaphragmatic hernias nor horseshoe kidneys were found. The kidneys of 33 control offspring were found to be normal in serial sections.

DISCUSSION

The experiments reported here represent an attempt to produce congenital malformations in the offspring of rats by maternal vitamin A deficiency. We intended to breed the females in a borderline state of vitamin A deficiency which would alter the development of the offspring without causing intrauterine death of the embryos. As this aim could be realized only in part, because sterility and intrauterine death on one side and normal development of the young on the other side interfered with the experiment, we attempted to regulate some of the factors which improve the yield of abnormal offspring. In our previous communication ('46) only 36 litters were obtained from 140 females. This corresponds to a fertility rate of 25.7%. In the present series the fertility rate was higher, since 114 of 260 females or 43.8% had litters. The increase in fertility rate resulted, however, in the birth of some normal offspring. It was shown in the experiments reported here that the character of the preparatory diet influenced the results to a certain extent. In general, one can expect that improvement of the dietary conditions will result in an increased fertility rate and in a lower percentage of abnormal offspring. While abnormalities of the same type can be obtained in the offspring of mothers who receive any

of the preparatory diets employed, it seems advisable to supplement diet U with 12–25 μg carotene every tenth day.

SUMMARY

Congenital abnormalities can be induced in the offspring of female rats reared and bred on diets deficient in vitamin A. The abnormalities are found in soft tissues and are different from those induced in the skeleton by maternal riboflavin deficiency.

Variations of the preparatory diet influence the yield of abnormal young. Increase of the carotene supplement results in an increased fertility rate, but the percentage of abnormal young decreases. Reduction of the carotene supplements reduces fertility while the percentage of abnormal young increases.

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THE NUTRITION OF THE MOUSE

II. EFFECT OF DIET ON THE BACTERIAL FLORA OF THE INTESTINE AND THE CECUM [1]

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It is well-known that diet influences the intestinal flora of several species of animals, as is shown by the work of Tissier ('08) and later Sanborn ('31) with humans, Belonovsky ('07) with mice, Herter and Kendall ('09–'10) with cats and monkeys, Hull and Rettger ('15, '17) and Porter and Rettger ('40) with rats, and Crecelius and Rettger ('43) with guinea pigs. In general, all of the species responded similarly whenever either of 2 types of diets was fed. Meat or a high protein diet produced proteolytic flora consisting largely of coliform organisms; a variation in the carbohydrate induced a change toward a fermentative flora. For example, milk or lactose, and sometimes dextrin, encouraged an aciduric bacterial population; when dextrin was fed, there was produced a type of flora which synthesized some of the B vitamins (Guerrant et al., '35; Schweigert et al., '45).

Belonovsky ('07) studied only the effect of lactose and milk on the bacteria of the feces and upon the ease of implantation of certain strains of bacteria in the intestine. Little attention was paid to the effect of varying the diet on the predominating

[1] Supported in part by the Nutrition Research Fund of this laboratory and in part by a grant from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council. The early experiments in this study were supported by grants from the Nutrition Foundation, Inc., and the Anna Fuller Fund.

flora or the effect that such a change would have in the nutrition of the mouse.

It has been shown in this laboratory that considerable difference exists in the nutritional requirements of several highly inbred strains of mice. In general, the requirements of the C_{57} strain (black, with low incidence of spontaneous mammary tumors) are lower than those for the A strain (albino, highly susceptible to spontaneous mammary tumors). Both of these strains have been used in these studies of the intestinal flora, but this paper deals principally with the A strain.

The studies to be reported here were undertaken to determine the effect of 2 synthetic rations and a natural diet upon the predominating bacteria in the intestinal tract of A strain mice, and whether any such change could affect the nutrition of the host. The lower parts of the small intestine and the cecum were chosen for investigation since it was felt that these were probably the most likely sites of absorption by the mouse of any synthetic products liberated by the bacteria.

The predominating bacteria of these segments of the intestine were studied by Gram stains and by culturing serial dilutions of the contents. Direct bacterial counts were made on the samples and the entire cecal contents were weighed.

EXPERIMENTAL

Weanling mice of the A strain, 21 days old, were placed in individual screen-bottom cages, and food and water supplied *ad libitum*. Growth rates were measured by weighing the animals periodically. The stock ration [2] and 2 synthetic diets (nos. 101 and 133) were used. The composition of the synthetic rations has been described in detail elsewhere (Fenton and Cowgill, '47). Diet 101 contained 23% casein and 60% dextrose, while diet 133 contained 30% casein and 45% dextrin. In other respects the 2 diets were similar except that no. 133 also had cystine, vitamin K, biotin and folic acid added.

Representative animals from these 3 groups were killed by etherization and were opened immediately using sterile tech-

[2] Purina Laboratory Chow.

nique. The cecum and the lower half of the small intestine were removed and the contents from these segments squeezed directly into sterile, tared vials which were then plugged with cotton. The upper part of the small intestine was not studied because of the small numbers of organisms present. The large intestine was not considered to be a likely area of absorption of bacterial excretory products because on the synthetic diets this material formed into hard pellets immediately upon leaving the cecum. It seemed likely that any substances that might be produced by bacteria in this region would be lost to the host. The vials containing the samples were weighed and the samples made up to a known dilution with sterile distilled water. The vials containing the diluted samples (usually a 1 : 25 to 1 : 100 dilution, depending on the size of the sample) were stoppered and shaken thoroughly. Any clumps of contents were broken up with a sterile pipette. The samples were then further diluted to 10^{-4}, and then serially by steps of 100 until a dilution of 10^{-10} was reached.

The cultural work was done first, and the whole procedure after the removal of the contents from the body was done as quickly as possible in order to keep the sample from being exposed unduly to air and low temperatures. The cecal contents were cultured first, as the bacteria isolated from this segment seemed to be more anaerobic than those from the small intestine. Attempts to reduce dissolved air to a minimum consisted of plugging the water blanks with rubber stoppers immediately upon removal from the autoclave, and of boiling the broth for 10 minutes in a water bath, followed by immediate cooling in water just before inoculation. The inoculum was placed carefully under the surface of the broth with a pipette, and the tube was sealed with vaspar.[3] The broth was composed of 1% of each of the following: Bacto-peptone, tryptone, peptonized milk, yeast extract, beef extract, and glucose, and 0.5% of K_2HPO_4 all dissolved in liver extract made by extracting 75 gm of Bacto liver powder in a

[3] Composed of equal weights of petrolatum and paraffin.

liter of distilled water. The pH was adjusted to 6.8 before autoclaving.

For the cecal cultures, inocula representing 10^{-8}, 10^{-9}, and 10^{-10} were put into the broth in duplicate while for the small intestine, only dilutions 10^{-8} and 10^{-9} were cultured. The cultures were incubated for 1 week at 37°C. Only these higher dilutions were cultured, as this study was primarily concerned with the predominating organisms. However, in order to learn more about the distribution of coliform organisms, in most cases the E C medium of Hajna and Perry ('43) was inoculated with dilutions of the samples from 10^{-2} to 10^{-7}.

As soon as growth appeared in any tube, the culture was Gram stained, the motility was determined, the pH of the broth was measured electrometrically, and agar shake cultures were made to indicate the degree of anaerobiosis and to permit purification if necessary. The agar shakes were saved so that certain synthetic activities of these cultures could be studied. The results of this phase of the work will be presented in the following paper (Gall et al., '48).

Immediately after the completion of the cultural procedure, a Gram stain was made of the diluted material from the original vial. From this the predominating morphological types and their approximate relation to each other were noted. Direct counts were also made on the sample after suitable dilution. In general the cecal content was diluted 1 : 1000, while the small intestinal content was diluted 1 : 25 to 1 : 100, depending on the expected bacterial count. The slide counts were made on 2 × 2 cm areas by mixing 0.01 ml of the diluted sample with 1 drop of saturated nigrosine solution in methyl alcohol spread evenly over the area. The slides were dried very quickly on a hot plate, and the counts were made with a microscope for which the field had been calibrated. It was easy to see the unstained bacteria against the black background, and from the average of 10 fields on each of 2 slides (about 300–400 organisms), it was possible to calculate the bacterial count per gram of original sample.

The weight of the cecal and small intestinal contents was determined to the nearest milligram on an analytical balance. The material came out of the cecum very easily, and it is felt that the weights obtained represented with fair accuracy the entire cecal contents. The weights of the contents of the small intestines were extremely variable, since the position of the lumps of food in the intestine was so irregular that the inclusion or exclusion of 1 mass of food would alter the result severalfold. Therefore not much significance is attached to these figures, and they are not reported in the table.

In addition to these main experiments on A strain mice a few preliminary studies were carried out on small groups of C_{57} and A strain mice on various other diets under investigation in the laboratory.

RESULTS

Growth rates of male mice fed the stock ration or diet 101 have been reported by Fenton et al. ('47). All 3 diets studied here supported growth about equally well. Fenton and Cowgill ('47) have reported complete failure in securing reproduction and lactation in mice raised and maintained on ration 101. The more complete diet 133 did support reproduction and lactation, but the results were still not as satisfactory as with the stock ration.

Gram stains of the cecal contents of the animals fed these 3 diets were examined microscopically, and the differences in the flora on these diets were sufficiently marked to permit identification of the diet fed, simply by studying the slide. The cecal contents of the animals on stock ration were characterized by the presence of a slender, gram-negative, curved rod and a cigar-shaped organism. Except for some gram-positive rods, probably lactobacilli, there were few other bacteria present. In contrast to this simple flora, the animals on diet 101 harbored a varied bacterial population, but there was always present in these animals a considerable number of large round cocci, occurring in pairs and short chains. This coccus was characteristic of this diet. Diet 133 also supported

a variety of organisms, but the flora differed from that on ration 101 in several ways. The large coccus found on diet 101 was absent, but in its place was found in smaller numbers an elongated coccus, occurring in pairs, and a tiny coccus forming a characteristic, closely united chain of 10–20 organisms. On diet 133 there also appeared in large numbers, a highly curved rod that formed a perfect C.

The Gram stains of the small intestinal contents from animals fed all 3 diets showed the gram-positive rod as the predominating bacterium, but on the 2 synthetic diets the coccus characteristic of that ration usually appeared.

A more complete description of the types of bacteria found on these various diets, as well as some notes on their synthetic activities, especially in regard to B vitamins, will be presented in the following publication.

As shown in table 1 the cultural differences among the 3 groups were also marked and largely confirmed the conclusions drawn from the examination of the slides. The cecal samples cultured from stock diet animals gave scanty growth, due to the predominance of the slender curved rod, an anaerobe which proved very difficult to culture. In no case did the cultures from the animals on this diet grow in as high a dilution as was expected from the slide count. Virtually the only organism isolated from these animals was the easily cultivatable gram-positive rod.

In contrast, the bacteria from the ceca of animals fed diet 101 grew very readily. In 9 out of 12 cases the cultures grew in the dilutions indicated by the slide counts. The characteristic coccus was easily isolated, as were the gram-positive rods, and the coliform organisms when the latter were present. Gas, usually found in 1 or more tubes in the series, could in some instances be attributed to coliforms. However, in some tubes only a slight amount of gas appeared. On staining these cultures only cocci were observed, and attempts to isolate coliforms from these tubes repeatedly failed. This cultural picture identified the animals which had been fed diet 101.

TABLE 1

Summary of the bacterial counts, weights of cecal contents and cultural results obtained from A strain mice.

DIET NUMBER	101		133		STOCK	
Part of intestinal tract	Small	Cecum	Small	Cecum	Small	Cecum
No. of animals	12	12	12	12	9	9
No. of animals showing agreement of slide and cultural "counts"	12	9	10	3	9	0
Wt. of segment contents, *gm*	[1]	0.065 ± 0.021 [2]	[1]	0.091 ± 0.029 [2]	[1]	0.107 ± 0.033 [2]
Bacterial count — billions						
per gm of sample	0.710 ± 0.81 [2]	22.2 ± 11.5 [2]	0.321 ± 0.26 [2]	23.0 ± 11.8 [2]	0.875 ± 0.99 [2]	12.8 ± 11.5 [2]
per intestinal segment	[1]	1.5 ± 0.84 [2]	[1]	2.09 ± 1.15 [2]	[1]	17.9 ± 5.4 [2]
No. of cultures isolated; according to type						
Gram + rods						
10^{-8}	14	7	9	10	13	13
10^{-9}	7	6	3	5	8	8
10^{-10}	[1]	3	[1]	0	[1]	0
Round coccus						
10^{-8}	7	15	0	0	0	1
10^{-9}	3	9	0	0	0	0
10^{-10}	[1]	5	[1]	0	[1]	0
Elongated coccus						
10^{-8}	0	0	1	6	0	0
10^{-9}	0	0	0	3	0	0
10^{-10}	[1]	0	[1]	0	[1]	0
Tiny coccus						
10^{-8}	0	0	1	3	0	0
10^{-9}	0	1	0	4	0	0
10^{-10}	[1]	1	[1]	1	[1]	0
Curved rod						
10^{-8}	1	0	1	1	0	1
10^{-9}	0	2	0	6	0	1
10^{-10}	[1]	3	[1]	1	[1]	0
Coliforms						
10^{-8}	0	4	0	3	0	0
10^{-9}	0	3	0	0	0	1
10^{-10}	[1]	3	[1]	0	[1]	0
No. of animals showing coliform organisms 100 M or above	[1]	5	[1]	2	[1]	1

[1] Not computed. [2] Standard deviation.

The cecal contents from animals fed ration 133 gave cultural results somewhat different from the other 2 diets. In only 3 cases out of 12 did the cultures grow in the dilution expected from the slide counts, but the discrepancy was not as great as with the stock ration. The elongated coccus and the tiny coccus, which proved to be an anaerobe, occurred in a fair number of cultures. The gram-positive rods were also isolated regularly. The curved, C-shaped rod seen on the smears was never isolated in that morphological form.

It has commonly been thought that coliform bacteria are always among the predominating organisms in the intestinal tract, but this study indicates that with A strain mice its occurrence in large numbers varies with the diet. With the average bacterial count in the neighborhood of 22 billion per gram of cecal contents, coliforms were isolated in dilutions of 1 billion or more only 7 times. Three of these came from the 10 billion dilution and 4 from the 1 billion. These cultures represented 5 animals, 4 of which were fed diet 101. Therefore, in only 10% of the animals tested were coliforms present in the top 2 dilutions, and in only 20% of the cases in the top 3 dilutions. It is pertinent to point out that 41% of the animals on diet 101 had coliforms present in 100 million or more, as compared to much fewer in animals on the other 2 diets. Two-thirds of the animals tested in low dilutions showed less than 1 million coliform organisms per gram of cecal contents and at least 25% were below 10 thousand per gram. It is of interest to note that with the stock ration animals where the opportunity for coprophagy was the greatest, there was the mildest coliform flora.

The cultural results of the small intestinal contents on the 3 diets reflected the findings with the Gram stain. All 3 diets gave a large number of cultures of gram-positive rods, while the 2 synthetic diets showed in addition cocci of the type characteristic of the ration.

The 3 diets were compared as to bacterial count per gram of cecal material and total bacterial count per cecum. For reasons stated above, a similar study for the small intestinal

contents is not reported in table 1. The animals eating the stock ration showed a higher bacterial content when measured per gram or per cecum, although the difference is greater by far when measured by the latter criterion. This is a function of the greater content of the cecum of the animals eating the natural diet. By statistical analysis these differences were found to be highly significant ($p < 0.01$). There was little difference between the animals eating the 2 synthetic diets, however. Statistically there was no difference between the cecal count per gram on diets 101 and 133. A comparison of the total cecal counts of these diets also showed no significant difference ($p = 0.2$).

The cecal contents obtained from animals fed the stock ration were statistically highly significantly heavier than the contents from the animals on the 2 synthetic diets ($p < 0.01$). The animals on diet 133 showed greater cecal contents than those on 101 (p just equals 0.01). This should be interpreted cautiously, however, for reasons to be discussed below.

By way of preliminary investigation a few animals of both C_{57} and A strains were studied after having been maintained on the following diets: (a) 3 diets similar to no. 133, but with dextrose as the carbohydrate, and a diet identical with no. 101 (containing dextrose), but omitting roughage; (b) a diet low in riboflavin and one low in pantothenate, but in other respects similar to no. 101; (c) a diet similar to no. 101 with 1% sulfathalidine added; (d) a ration identical with no. 101, but replacing the dextrose with dextrin.

The diets listed under (a) showed a cecal flora essentially the same as that found on ration 101. The animals deficient in riboflavin and pantothenate showed the same cecal flora as those on diet 101 except that a C-shaped rod was also present. The mice fed sulfathalidine showed a flora similar to those on ration 101, but the weights of their cecal contents were somewhat greater. When the dextrose of diet 101 was replaced with dextrin, the resulting flora resembled that which was found on the dextrin containing diet no. 133. In all of these preliminary studies it must be emphasized that only a micro-

scopic examination was made, thus affording a comparison on the basis of morphology alone.

A group of mice was fasted 24 hours and then fed the stock ration. Small numbers of these animals were sacrificed and their cecal contents studied at various intervals following feeding. The animals sacrificed 3.5 to 6 hours after feeding showed a C-shaped rod, which was not present in animals permitted to feed until sacrificed or in mice sacrificed up to 2.5 hours after feeding, following a period of fasting.

DISCUSSION

Ample evidence has been presented in the main portion of this study and also in the preliminary work reported here showing that the composition of the diet can have a marked effect upon the intestinal flora of the experimental animals. The bacteria seen on each of the 3 rations investigated have been so distinctive that it was possible to judge by microscopic examination alone which diet had been fed to any particular animal. In fact, following the initial experiments, we made it a practice to give the investigator (L.S.G.) an animal for the day's work without her having any knowledge of the nature of the ration. On the basis of the Gram stain, the examiner would attempt to identify the diet fed, and in only 1 case out of 33 was an error made.

Many investigators have implied that a great deal of the intestinal synthesis should be ascribed to coliform bacteria. Our data do not warrant such conclusions, at least for this highly inbred strain of mouse. We have found appreciable numbers of coliforms only on diet 101, and here only 5 out of 12 animals showed this organism in the top dilutions.

It is important to stress that with the animals fed the stock ration and diet 133, we frequently failed to obtain cultural results commensurate with the slide count. More work is necessary to determine the cultural requirements of the bacteria which predominate on this diet.

In view of the greater roughage content of the stock ration, it was to be expected that animals on this diet would have

greater cecal contents than the animals on synthetic diets. In our studies comparing 2 synthetic diets, 1 containing dextrin (no. 133) and the other dextrose (no. 101), there was a statistically significant difference between the weights of the cecal contents of the 2 groups (p just equals 0.01). However it must be stated that the animals fed diet 133 were slightly older than those eating no. 101, and for this reason the results should be interpreted with caution. Mannering et al. ('44) found that rats fed a synthetic diet containing dextrin had a larger cecal content per unit of body weight than animals fed a similar diet containing sucrose.

It must also be stated in this connection that 6 of the 9 mice fed the stock ration were older than the animals on synthetic ration, while 3 were the same age or younger. On comparison the average weights of the cecal contents of the young and old animals on stock ration were found to be so nearly alike ($0.386 \pm .038$ and $0.417 \pm .045$, respectively), that it was felt that these age differences did not seriously affect the interpretation.

The study reported here on both C_{57} and A strains of mice on different diets gives evidence that there is a flora, characteristic of mice fed a dextrose diet, which is different from that of animals maintained on a ration containing dextrin. This is in agreement with the findings of other workers on several species cited in the introduction, that certain carbohydrates induce differences in intestinal flora.

SUMMARY AND CONCLUSIONS

1. Microscopic and cultural examinations of the small intestine and the cecum of A strain mice on stock ration and 2 synthetic diets were carried out.

2. Mice fed each of these diets had a characteristic cecal flora; on the stock ration it consisted of a slender, gram-negative, curved rod; on the 2 synthetic diets cocci characterized each of them.

3. Coliforms were not found to be present in significant numbers on 2 of the diets and were found in large numbers in less than half of the mice on diet 101.

4. The mice on the stock diet had a larger bacterial population per gram of cecal contents and per cecum than the mice on synthetic diets. There was no significant difference between the 2 groups of mice on synthetic diets.

5. The weights of the cecal contents of the animals on stock ration were greater than those of the animals fed synthetic diets. There was little difference between the animals on synthetic diets with respect to weights of cecal contents.

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THE NUTRITION OF THE MOUSE

III. RELATION OF DIET TO THE SYNTHETIC ACTIVITY OF THE PREDOMINATING FLORA ISOLATED FROM THE SMALL INTESTINE AND CECUM [1]

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It has been established that the bacteria of the intestinal tract are active in the synthesis of certain B vitamins (Mitchell and Isbell, '42), and it has been demonstrated that the cecum is probably the site of this synthesis (Taylor et al., '42; Schweigert et al., '45). It has also been shown that certain carbohydrates such as dextrin promote greater intestinal synthesis of these B vitamins than the simpler sugars, sucrose and dextrose (Guerrant et al., '35; Schweigert et al., '45).

In most of these studies, the synthetic activities of the whole intestinal flora as found *in vivo* have been investigated, with little attempt to isolate and study the synthetic activities of the predominating flora. Also since it is the vitamin content of the fecal flora that has been the subject of several studies, it has not been clearly demonstrated whether the vitamins are available to the host, unless coprophagy is practiced or considerable autolysis occurs in the intestinal tract.

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The purpose of our preliminary studies has been to determine whether any of the predominating organisms isolated from the small intestines and ceca of mice fed different diets showed any marked differences in their synthetic activities with respect to the B vitamins, especially the occurrence of the vitamins in the medium surrounding the bacterial cells. We have observed in this laboratory (Fenton and Cowgill, '47a, b) that mice of the C_{57} and A strains differed in their requirements for riboflavin and pantothenic acid. It seemed to us that possibly this difference could be attributed to differences in the rates of intestinal synthesis.

EXPERIMENTAL

The methods for maintenance of the mice and for isolation of the predominating flora have been presented in the preceding paper (Gall et al., '48). A description of the diets or a reference to their composition will also be found in that paper.

The cultures were obtained from 62 mice of the A and C_{57} strains fed 7 different diets, which can be grouped into 3 main classes: a stock diet and 2 synthetic diets, 1 containing dextrin and the other dextrose. This study included the testing of a total of 107 cultures, of which 83 came from the 3 top dilutions of the cecal contents, 18 from the 2 highest dilutions of the small intestinal contents, and 6 were coliform organisms from lower dilutions of contents from both intestinal segments.

The organisms were tested within 1 week of isolation in most cases. Cultures for testing were obtained by planting a single colony from an agar shake culture prepared from the original broth into a complete synthetic broth (Krehl and Illingworth), modified by using the casein hydrolysate described by Roberts and Snell ('46). Since most of the original cultures showed only 1 type of bacterium in the Gram stain, little difficulty was experienced in obtaining a pure culture. In those few cases where a tested organism came from a mixed culture, a series of agar shake cultures was made to insure a well-isolated colony. To check for purity, Gram stains

were made from the broth planted with the single colony, and from the culture representing the third transfer in complete broth.

After the culture in complete broth had been incubated for 24 hours at 37°C., it was observed for growth. If turbidity was apparent, 0.1 ml of the culture was used as an inoculum for the various broths employed to test its synthetic abilities. The media inoculated were as follows: a complete synthetic broth of the same composition as that in which the organism had been growing, and 5 deficient broths, identical with the complete broth, except for the omission of 1 of the following vitamins from each broth — riboflavin, niacin, biotin, folic acid and pantothenic acid.

All broths for the series of 3 transfers and the subsequent microbiological assays on these cultures were prepared simultaneously once each week. Three serial transfers were made of each culture at 24-hour intervals, each culture being transferred into a broth identical with the one on which it was growing. Immediately prior to the inoculation with 0.1 ml of the culture, the broths were autoclaved and cooled rapidly. All anaerobic cultures were sealed with vaspar. The growth of the cultures was graded from 1 + to 4 +, indicating the degree of turbidity produced. If a tube failed to show visible growth before the third transfer, it was carried for one more transfer before being discarded.

A slightly different procedure was used for the coliforms after it became apparent that the synthetic broth referred to above would not support satisfactory growth of the coliforms. A mineral salt and glucose broth (MacLeod, '40) was used for the serial transfers.

If the culture survived 3 transfers in any of the deficient broths, the supernatant fluid obtained by centrifuging the 24-hour culture was tested for the presence of the omitted vitamin. This was done by microbiological assay, following the procedure of Snell ('47) except for a modification of the broth. The synthetic medium used in this study was the broth of Krehl and Illingworth (unpublished data), modified as

noted above. The assay was carried out on 1, 2 and 4 ml of the supernatant broth, using *L. casei* as the test organism for riboflavin and folic acid, and *L. arabinosis* for niacin, biotin and pantothenic acid. No regular standard curve was run — only a blank and a maximum tube — as it was not desired to estimate the amount of B vitamin quantitatively, but merely to determine whether large or small amounts of the vitamin were liberated from the cells. The results were graded on a scale running from 1 + to 4 +. The 1 + and 2 + values were interpreted as indicating slight synthesis and liberation of the vitamin, while 3 + and 4 + values meant that the titration approached or equalled that of the maximum tube, indicating considerable amounts of the vitamin to be present in the broth. A value was not considered significant unless the titration of the tube containing 1 ml of the supernatant fluid was greater than the blank, and unless the titration increased as the amount of the added supernatant fluid increased.

RESULTS

In general the predominating organisms isolated from the intestinal contents of the mice on the different diets were of 7 types. A table showing the distribution of 6 of these types isolated from A strain animals on different diets will be found in the preceding paper (Gall et al., '48). The seventh type occurred mostly in 10 out of 12 black mice on diet 101, and will be discussed here. The distribution of the other organisms from the C_{57} mice was about the same as that for the white mice on the same diets.

A summary of a few characteristics of the bacteria isolated is presented in table 1. None of the organisms isolated was motile or formed spores, so these characteristics were not included in the table. No attempt has been made to key out these bacteria, but some tentative genus names will be suggested for 3 of the types of organisms. The gram-positive rods were probably Lactobacilli, and the gram-negative anaerobic rods, both the curved and the short fat ones, resembled certain of the Bacteroides as described by Eggerth and Gag-

TABLE 1

Some characteristics of 7 types of bacteria isolated from high dilutions of small intestinal and cecal contents of mice on several different diets.

ORGANISM TYPE AS DESIGNATED IN TEXT	MORPHOLOGY	GRAM REACTION	OXYGEN RELATION	GROWTH IN ORGANIC BROTH	FINAL PH IN ORGANIC BROTH	GAS PRODUCTION	GROWTH IN SYNTHETIC BROTH	DISTRIBUTION ON VARIOUS DIETS
Gram-positive rod	single or short chains	positive	facultative	moderate 16 hrs.	5.5–6.0	negative	poor	all diets
Curved rod	single or pairs	negative	anaerobic	slight 3–4 days	6.3–6.4	negative	poor	all diets
Short, fat rods	single	negative	anaerobic	moderate 2–3 days	6.0–6.3	negative	absent	mostly black mice on diet 101
Coliforms	single	negative	facultative	heavy, 16 hrs.	5.3–5.4	heavy	poor	mostly white mice on diet 101
Tiny coccus	long, closely united chains	positive	anaerobic	slight 2–3 days	5.9–6.0	negative	absent	mostly diet 133; few on 101
Round coccus	short chains and masses, pairs	positive	facultative	heavy, 16 hrs.	4.5–4.7	slight?	good	dextrose containing diets
Elongate coccus	pairs and long chains	positive	facultative	heavy, 16 hrs.	4.9–5.3	negative	good	dextrin containing diets

non ('33). The gram-positive organisms usually exhibited that reaction only in young cultures, and the cocci frequently appeared gram-negative in the original stain from the intestinal contents. The overgrowth of the slower growing anaerobes by the faster growing bacteria explains the occurrence of these anaerobes in the top dilution only, where they are found after the competing organisms have been diluted out.

These 7 types of bacteria were tested for their ability to survive 3 transfers in the absence of certain of the B vitamins omitted singly from an otherwise supposedly complete broth, and if they survived, to contribute to this broth measurable amounts of the vitamin omitted, as shown in table 2.

It will be observed from this table that 2 of the types of organisms never grew in the synthetic broth, and therefore their synthetic activities could not be tested. Two more of the bacterial types grew poorly when they survived at all. The curved rod was subcultured only twice, but both times it survived the 3 transfers in the pantothenic acid deficient broth, and was shown to liberate this vitamin into the broth, in 1 case in large quantities. The gram-positive rods that grew readily in the organic medium proved difficult to culture in the synthetic broth, and although one-third of the trials to obtain 3 transfers in synthetic broth were successful, the growth was weak. In a few cases small amounts of one of the B vitamins were found to be present in the broth. The poor growth might account for the slight synthetic activity observed.

Oddly enough the coliforms grew poorly in this synthetic broth. Known strains of E. coli [2] as well as the recently isolated cultures, gave correspondingly poor results. When the simpler, mineral salt broth was employed, growth was better, and the supernatant broth of these cultures was found to contain considerable quantities of all 5 vitamins.

A striking difference appeared when we compared the activity of the 2 facultative cocci. They both grew well in the synthetic broths, both complete and deficient, with the ex-

[2] Obtained through the kindness of Dr. P. B. Cowles, Department of Bacteriology, Yale University.

TABLE 2

Summary of the ability of 7 groups of intestinal bacteria to synthesize and liberate 5 B vitamins.

TYPE OF ORGANISM	NO. OF CUL-TURES TESTED	NO. SUR-VIVING 3 TRANS-FERS IN COM-PLETE BROTH	INTENSITY OF GROWTH IN BROTH	NO. SURVIVING 3 TRANSFERS IN BROTH DEFICIENT IN						DEGREE OF LIBERATION OF VITAMIN	NO. OF TIMES VITAMIN FOUND IN SUPERNATANT BROTH				
				Complete	B_1	Niacin	Biotin	Folic acid	Panto-thenic acid		B_1	Niacin	Biotin	Folic acid	Panto-thenic acid
Gram positive rod	32	11	+	11	11	1	11	7	8	+	1	0	2	2	0
Curved rod	12	2	+	2	1	1	0	0	2	+	0	0	0	0	1
										+++	0	0	0	0	1
Short fat rod	4	0													
Tiny coccus	7	0													
Round coccus	30	28	+ to ++	15	18	21	16	6	14	+ to ++	7	2	2	3	1
			+++ to ++++	13	7	0	4	3	4	++++	0	0	0	1	0
Elongate coccus	8	8	+ to ++	3	6	8	5	3	6	+ to ++	4	1	2[1]	0[1]	0
			+++ to ++++	5	2	0	3	5	1	+++ to ++++	0	0	0	6	0
Coliforms	14														
regular synthetic broth	4	3	+ to ++	3	2	3	2	3	2	+ to ++	1	3	0	0	0
										+++ to ++++	1	0	0	2	0
mineral salt broth	10	9	+++	9	(mineral broth only)					+ to ++	4	5	3[1]	0	2
					no vitamins added					+++ to ++++	1	1	3	9	5

[1] One culture in group spoiled while testing.

ception of the medium without folic acid. In this broth the coccus from animals on diets containing dextrin grew as well as on the other synthetic broths, while only 9 out of 28 cultures of the coccus from the mice fed dextrose-containing diets survived the 3 transfers in this medium, and 6 of these showed only weak growth. This difference is further emphasized by noting the data on the presence of folic acid in the supernatant broth after the cocci had been planted in the folic acid deficient broth. Of the 7 cultures tested from animals fed diets containing dextrin, 6 showed the presence of large amounts of folic acid in the supernatant broth. In contrast, the cocci from mice fed dextrose-containing diets, showed liberation of folic acid in only 4 cultures, and of these, only 1 showed the presence of large amounts.

DISCUSSION

The results of this preliminary study tend to indicate that differences in diet influenced the bacterial synthesis of B vitamins by the alteration of the organisms present in the intestinal tract. However, this is far from the final answer, as will be pointed out in the following discussion.

There are 2 major weaknesses in this work. The first, which is largely unavoidable with our present culturing techniques, is the valid criticism that the *in vitro* findings do not necessarily reflect the *in vivo* activities of the organism. The second weakness lies in the inability to isolate some of the predominating organisms at all, or to subculture a bacterium in synthetic broth even after primary isolation, thereby making the study of their synthetic activities impossible. Obviously further work is needed on this aspect of the problem.

This indicates that in this study, the techniques employed failed to meet the cultural requirements of all of the predominating flora. This proved to be especially true of the cecal bacteria. The conditions were more nearly met with the rich organic broth than with the synthetic medium, however, as was shown by the inability to subculture some of the primary isolations in synthetic broth, although they could be success-

fully transplanted into organic media. This suggests that for these bacteria, the synthetic broth may have had a deficiency or an imbalance.

However, the poor growth or lack of growth of some organisms in the rich organic medium indicates that there are still other flaws in the present technique. This may well be remedied by improving the means of maintaining anaerobiosis, as many of the cecal organisms were shown to be obligate anaerobes. Further work should be done in the field of culturing these organisms.

Several considerations, to be discussed here, entered into the development of the technique as it was used in these studies. In the preceding paper we have discussed our reasons for choosing the lower small intestine and the cecum for our study of their flora, since it would seem that in these areas any vitamins liberated from the cells would have the best chance of being absorbed by their host. It was also because of considerations of availability to the host that we selected the supernatant fluid of the cultures to test for the presence of the vitamins. Any vitamins held in the cells would be available to the host only if he practiced coprophagy, or if active autolysis took place in the intestinal tract at or above a point where absorption could take place. As the degree of cellular breakdown which takes place in such a region is uncertain, we tried to minimize autolysis as much as possible by using young cultures (24-hour) for testing. In most cases our cultures were increasing in turbidity between the eighteenth and twenty-fourth hour, suggesting an actively growing culture rather than a static one in which autolysis would be prevalent.

In these studies we have considered only the predominating flora of the cecal and small intestinal contents, as we felt that the bacteria present in the largest numbers would have the greatest chance to make a significant contribution to their host by any liberation of vitamins.

As has been pointed out in the preceding paper, we have not regularly found coliforms to be present as a predominating

organism except in a few white mice fed diet 101. In most cases these organisms appeared only at a much lower level of dilution. It does not seem likely that the coliforms, in spite of their good synthetic activities with respect to the 5 B vitamins tested (Thompson, '42; Burkholder and McVeigh, '42; also this paper), could contribute much to the nutrition of the mice maintained under the conditions of this study, with the exception of the few mice fed diet 101, mentioned above.

The relationship of the presence of coliforms to diet is only 1 instance of the role that diet plays in influencing the type of intestinal flora of the mouse. The difference in the type of cocci isolated from mice fed dextrose- or dextrin-containing diets is further evidence of the effect of the carbohydrate in the ration on the intestinal bacteria isolated. The importance of this difference in cocci is brought out further by the striking difference in their synthetic activities with respect to folic acid. From these results, we can assume that the coccus isolated from animals on a dextrin-containing diet was probably supplying folic acid to its host. In contrast, the coccus isolated from mice maintained on a diet containing dextrose was not elaborating any significant amount of folic acid, but apparently actually required it for growth. It was therefore probably competing with its host for any folic acid present in the diet or liberated from any other intestinal organism. Such competition for this essential nutrient might even prove detrimental to the host.

Riboflavin was found to be present in small amounts in the supernatant fluid of about one-fifth of the cultures tested. Since it has been shown (Mitchell and Isbell, '42) that riboflavin is not readily liberated from the cell, this is in keeping with the work of other investigators in this field. Although the amount liberated by each organism was small, it may be enough to become of importance when an animal is on a marginal riboflavin intake. Also any factor which would increase the number of these organisms, such as an increase in bacterial population in the cecum, should correspondingly increase the total amount of riboflavin produced. The difference in the ribo-

flavin requirement between the C_{57} and A strain mice (Fenton and Cowgill, '47a) might be explained by the difference in bacterial population in the cecum (Gall et al., '47). A similar explanation might apply to the differences in requirements of these 2 strains for pantothenic acid (Fenton and Cowgill, '47b), since the curved rod which is a predominating organism on all 3 diets, liberated pantothenic acid both times it was successfully cultured in synthetic broth.

On the basis of these preliminary studies, it might be said that diet may influence the nutrition of the host indirectly by affecting the intestinal flora, as well as by supplying the nutrients directly.

SUMMARY AND CONCLUSIONS

1. A study was undertaken to determine the ability of 107 cultures, isolated from high dilutions of intestinal contents of mice fed 7 different diets, to synthesize and liberate from their cells 5 vitamins — riboflavin, niacin, biotin, folic acid and pantothenic acid, and to determine whether the synthetic products of the flora were influenced by diet.

2. A partial description was given of 7 different types of bacteria found in the top dilutions of intestinal contents of mice, with a notation as to their distribution on the various diets.

3. Differences were observed in the ability of these cultures to grow in synthetic broths, both complete and deficient in 1 of the 5 vitamins. In some instances these differences were correlated with diet, as in the case of 2 cocci. The coccus characteristic of the flora found in mice fed dextrin-containing diets, grew well in the synthetic broth lacking folic acid, in contrast to the coccus found in animals fed a diet containing dextrose, where little or no growth was observed in the folic acid-deficient broth.

4. These 2 cocci also exhibited differences in the extent of folic acid liberation; the coccus which grew well on the broth lacking folic acid liberated large amounts of this vitamin into the environment, while the coccus which grew poorly, or not

at all, in the folic acid-deficient broth, liberated little if any of this vitamin.

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EFFECTS OF B VITAMINS, LIVER AND YEAST ON GROWTH UNDER COLD ROOM AND ROOM TEMPERATURE CONDITIONS [1]

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Available data indicate that liver contains at least 1 factor other than the known B vitamins essential for optimal growth in the immature rat (Bosshardt, Ayres, Ydes and Barnes, '46; Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47; Cary, Hartman, Dryden and Likely, '46). Weanling rats fed purified rations containing liver invariably gained more weight than animals fed similar diets containing the B vitamins in synthetic form. These differences, although consistent for all experiments cited above, were not sufficiently marked in most cases to be statistically significant (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47), particularly with diets containing unextracted casein (Cary, Hartman, Dryden and Likely, '46). The present experiment was undertaken to determine whether the above differences in growth might not be accentuated by raising animals under conditions of cold. Available data indicate that body requirements for essential nutrients may be increased by physical exertion,

[1] The research which this paper reports was undertaken in cooperation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

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fever, thyroid-feeding and other conditions resulting in an increased metabolic rate (Ershoff, '48). Such "stress factors" raise body requirements beyond the usual or average range, accentuate deficiencies and hasten the onset of symptoms on a deficient diet. It was felt that since excessive doses of thyroid caused retarded growth preventable by liver feeding in the rat (Ershoff, '47a, '47b), exposure to cold with its attendant rise in oxygen consumption might also serve as a "stress factor" resulting in increased requirements for unknown growth factors in the rat.

PROCEDURE AND RESULTS

Four basal rations were employed in the present experiment: diets A, B, C and D. Diets A and B were purified rations containing the B complex factors in synthetic form only. Diets C and D were similar in composition but contained yeast or desiccated whole liver in addition to the synthetic vitamins. Ninety female rats of the Long-Evans strain were selected at 23 to 25 days of age at an average weight of 55 gm. Animals were kept in metal cages with raised screen bottoms to prevent access to feces and were fed *ad libitum* the diets listed in table 1. Feeding was continued for 8 weeks. Experiments were conducted (1) with animals kept continuously in a large walk-in refrigerator at a temperature of 2 ± 1.5°C.[3] and (2) under standard laboratory conditions at an average temperature of approximately 23 ± 2°C. Food consumption was determined daily for the first 4 weeks of feeding. Animals were autopsied after 8 weeks of feeding, organ weights determined, the ovaries and thyroids fixed in 10% formol, and sections prepared and stained with hematoxylin and eosin. A second experiment was conducted under conditions similar to the above except for a cold room temperature of 6 ± 2°C. in contrast to the 2 ± 1.5°C. employed above. Forty-eight

[3] Animals were kept continuously in the dark except for such times as they were being fed or weighed. Previous work indicates that exclusion of light is without significant effect on resulting pathology under cold room conditions (Kenyon, '33).

female rats of the Long-Evans strain were weaned at 21 to 23 days of age and fed diets A, C and D, both under cold room and room temperature conditions. Feeding was continued for 8 weeks. During the eighth week of feeding metabolic rates were determined for all rats in both the cold room and room temperature series.

TABLE 1

Composition of experimental diets.

DIETARY COMPONENT	DIET A	DIET B	DIET C	DIET D
Yeast [1]	0.0	0.0	10.0	0.0
Whole liver powder [2]	0.0	0.0	0.0	10.0
Vitamin test casein [3]	22.0	22.0	22.0	22.0
Salt mixture [4]	4.5	4.5	4.5	4.5
Sucrose	73.5	73.5	63.5	63.5

To each kg of diet A, C and D were added the following synthetic vitamins: thiamine hydrochloride 72 mg, riboflavin 9 mg, pyridoxine hydrochloride 15 mg, calcium pantothenate 67.2 mg, nicotinic acid 60 mg, 2 methyl-naphthoquinone 5 mg and choline chloride 1.2 gm.

To each kg of diet B were added thiamine hydrochloride 144 mg, riboflavin 18 mg, pyridoxine hydrochloride 30 mg, calcium pantothenate 134.4 mg, nicotinic acid 120 mg, inositol 1.2 gm, p-aminobenzoic acid 600 mg, folic acid 10 mg, biotin 1 mg, 2-methyl-naphthoquinone 10 mg and choline chloride 1.2 gm.

Each rat also received 3 times weekly the following supplement: cottonseed oil (Wesson) 500 mg, alpha-tocopherol 1 mg, and a vitamin A–D concentrate [5] containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

[1] Brewers' Type Yeast no. 200, Anheuser-Busch, Inc., St. Louis, Mo.

[2] Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

[3] Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Salt Mixture no. 1 (Sure, '41).

[5] Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per gram.

Findings are summarized in table 2. Data for the cold room series were computed on the basis of the top 8 animals in each group (originally 12 rats per group) in the first experiment and for the top 7 animals per group (initially 10 rats per group) in the second experiment to minimize variations in averages due to early deaths, infection and atypical responses on the part of individual rats. Growth was significantly reduced in all rats under cold room conditions; animals fed

liver (diet D), however, gained significantly more weight than those fed synthetic rations (diets A and B) with growth on the yeast-containing ration (diet C) intermediate between the two.[4] At room temperature findings confirm those of earlier workers (Sporn, Ruegamer and Elvehjem, '47; Jaffé and Elvehjem, '47). Animals fed liver gained more weight than

TABLE 2

Effects of B vitamins, liver and yeast on growth of rats maintained under cold room and room temperature conditions.

GROUP DIETARY	FIRST EXPERIMENT			SECOND EXPERIMENT		
	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period[1]	Number of animals	Average initial body weight	Average gain in body weight over 8-wk. period[1]
Cold room series						
		gm	*gm*		*gm*	*gm*
A	8	54.4	88.4 ± 2.8	7	50.1	84.9 ± 3.1
B	8	56.0	93.0 ± 3.9			
C	8	55.6	99.3 ± 3.8	7	49.8	91.6 ± 3.7
D	8	56.0	111.5 ± 5.0	7	50.3	112.7 ± 4.8
Room temperature series						
A	6	53.8	146.0 ± 7.3	6	49.6	142.3 ± 8.1
B	6	53.9	155.4 ± 9.6			
C	6	53.7	159.0 ± 8.1	6	49.4	152.8 ± 8.5
D	6	53.7	167.7 ± 9.9	6	49.8	160.5 ± 10.2

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

those fed synthetic or yeast-containing rations. At room temperature, however, these differences were in no case sufficiently pronounced to be statistically significant.

Data on food consumption and the relative efficiency of the various diets for the building of body tissue are summarized in table 3. The findings indicate that for the first 4 weeks of feeding relative efficiency as measured by the ratio of the gain

[4] The highest values obtained on diet A were less than the average on diet D. In both experiments the quotient of M.D./S.E.M.D. for diets A and D was in excess of 4. Values of 3 or larger are considered statistically significant.

TABLE 3

Summary table showing average gain in weight, average food consumption and ratio of increase in weight to calories consumed for the first 28 days of feeding.

DIETARY GROUP	INITIAL BODY WEIGHT	AVERAGE GAIN IN BODY WT.[1]	FOOD CONSUMPTION (GM/DAY) ON FOLLOWING WEEKS OF EXPERIMENT[2] 1st	2nd	3rd	4th	AVERAGE TOTAL FOOD INTAKE PER RAT FOR FIRST 4 WEEKS OF FEEDING[2,3]		EFFICIENCY[4]
Cold room series (8 animals per group)									
		gm					*gm*	*calories*	
A	54.4	51.8 ± 2.3	12.7	14.8	15.5	16.9	119.6 ± 8.5	1552.5 ± 31.5	3.31 ± 0.18
B	56.0	54.0 ± 2.3	13.2	16.9	17.1	18.0	457.0 ± 9.2	1690.9 ± 34.0	3.28 ± 0.11
C	55.6	53.8 ± 3.4	12.8	15.5	16.2	17.9	435.2 ± 11.1	1610.2 ± 41.1	3.37 ± 0.17
D	56.0	63.7 ± 3.3	13.0	16.4	17.1	18.0	451.1 ± 12.3	1669.0 ± 45.5	3.88 ± 0.09
Room temperature series (6 animals per group)									
A	53.8	91.3 ± 4.6	8.9	10.5	11.6	11.7	298.8 ± 3.7	1105.6 ± 13.7	8.19 ± 0.41
B	53.9	104.7 ± 5.2	9.5	11.1	12.6	13.2	324.6 ± 12.8	1201.0 ± 47.5	8.70 ± 0.20
C	53.7	111.5 ± 5.0	9.6	11.6	12.5	13.0	325.8 ± 13.6	1205.5 ± 50.4	9.22 ± 0.28
D	53.7	120.2 ± 8.6	9.8	12.2	13.2	13.7	341.8 ± 16.1	1264.7 ± 59.2	9.45 ± 0.30

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

[2] These values are based on basal ration only and do not include the cottonseed oil ingested in supplementary feedings.

[3] The caloric value of diets A, B, C or D approximated 3.7 calories per gram of basal ration.

[4] $\frac{\text{Gm increase in weight}}{\text{Calories consumed}} \times 100$.

in weight × 100 to the calories consumed was slightly higher for animals fed liver (diet D) both under cold room and room temperature conditions than for those fed other diets tested. In most cases, however, these differences were not statistically significant. Values for the cold room series were less than for animals maintained at room temperature conditions. These differences were due, at least in part, to increased heat loss under cold room conditions.

TABLE 4

Body and organ weights of rats maintained under cold room and room temperature conditions.

DIETARY GROUP	INITIAL BODY WEIGHT	BODY WEIGHT AFTER 8TH WEEK OF FEEDING	VENTRICULAR WEIGHT	KIDNEY WEIGHT	OVARIAN WEIGHT	ADRENAL WEIGHT
Cold room series (8 animals per group)						
	gm	*gm*	*mg*	*gm*	*mg*	*mg*
A	54.4	142.8	785	1.678	35.4	40.0
B	56.0	149.0	733	1.634	37.0	36.6
C	55.6	154.9	770	1.832	32.5	39.2
D	56.0	167.5	826	1.868	40.3	51.1
Room temperature series (6 animals per group)						
A	53.8	199.8	640	1.602	46.1	46.0
B	53.9	209.3	659	1.664	52.7	44.2
C	53.7	212.7	637	1.673	55.5	49.7
D	53.7	221.4	649	1.758	54.3	59.5

At autopsy significant differences were observed between animals raised under cold room and room temperature conditions (table 4). Not only was growth retarded in the former series but approximately half the rats in this group lost part or all of their tails during the 8-week feeding period;[5] ventricular weight was increased not only relative to body weight but in absolute weight as well; kidney weights were increased relative to body weight; thyroids were similar to those described by Kenyon ('33) with cellular hypertrophy and loss of colloid; and approximately one-third of the rats

[5] Apparently due to poor circulation in the tail with resulting necrosis and sloughing off of the affected tissue.

had ovaries infantile both in weight and microscopic appearance. With the exception of growth, animals raised under cold room conditions did not differ significantly from one another on any of the diets employed.

Data on oxygen consumption for animals raised under cold room and room temperature conditions are summarized in table 5.[6] The apparatus employed in making these determinations was a closed circuit type with a capacity of 2 liters

TABLE 5

Oxygen consumption of rats maintained under cold room and room temperature conditions.

DIETARY GROUP	NUMBER OF ANIMALS	O_2 CONSUMPTION ML/HR/100 GM BODY WEIGHT[1]	INCREASE OVER B.M.R.[2]
		Cold room series	
			%
A	7	252 ± 17	92.4
C	7	205 ± 12	50.7
D	7	198 ± 8	55.9
		Room temperature series	
A	6	131 ± 5	...
C	6	136 ± 5	...
D	6	127 ± 4	...

[1] Including standard error of the mean calculated as follows: $\sqrt{\Sigma d^2/n}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

Values for O_2 consumption in ml/hr/100 gm body weight are somewhat greater for the room temperature series than those usually reported as basal for the rat. These differences may be due in part to a strain difference.

[2] Compared to the control B.M.R. in the room temperature series.

(Mason and Winzler, '47). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. Readings were made during the eighth week of feeding, and for the cold room series at a temperature of 6°C. with apparatus present in the same room in which animals were housed. In the room temperature series respiration chambers

[6] We are indebted to Mr. G. D. Mason for these determinations.

were kept at 28°C., and in both cases readings obtained were corrected to standard temperature and pressure. At least 6 successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum. Findings in table 5 indicate that average oxygen consumption in ml/hr/100 gm body weight was at least 50% greater for the cold room rats than were values obtained at 28°C. for the room temperature series.[7] In the cold room series values for diet A were greater than for rations containing liver or yeast; there is a question, however, whether these differences are significant. In the room temperature series no significant difference in metabolic rate was noted on the various diets employed.

DISCUSSION

Since the differences in growth between the synthetic and liver-containing diets were significant under cold room but not room temperature conditions, it is felt that the increased metabolism resulting from exposure to low environmental temperature increased requirements for 1 or more nutrients present in liver but not present in significant amounts in other diets employed. The increased requirements under cold room conditions accentuated tissue deficiencies on the synthetic diets employed and resulted in a gain in body weight significantly less than that observed when liver was added. Findings in the present experiment indicate that the protective factor was distinct from any of the known B vitamins and that it was not present in significant amounts in yeast. Liver, however, counteracted only part of the growth retardation observed under cold room conditions. This finding indicates either that (1) insufficient amounts of liver were employed, (2) the retardation of growth was due to factors other than of nutri-

[7] The increase in metabolism due to exposure to low environmental temperature was less in the present series than that reported by other investigators (Benedict and MacLeod, '29). This difference may be due in part to adaptation during our prolonged feeding period as well as to possible differences in the strain of rat employed.

EFFECT OF VEGETARIAN SELF-SELECTION DIETS ON REPRODUCTION AND THE GROWTH OF OFFSPRING OF RATS[1]

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In a preceding paper ('47), we described vegetarian self-selection diets (diets 6, 6C and 6CC) which were used in studies of the growth and longevity of rats. These diets differed only in regard to the fresh leafy vegetable supplements that were used. Diet 6 was supplemented mainly by lettuce, diet 6C by lettuce and celery cabbage, and diet 6CC by celery cabbage only. The present paper concerns the effects of these diets on the reproduction and growth of offspring of rats.

METHOD OF STUDY

First, the effect of diet 6 on the reproduction and growth of offspring of 3 generations of Wistar rats was determined. After that, the effect of diets 6C and 6CC on 3 generations of Wistar and Sprague-Dawley rats was determined. In these tests, the rats were inbred by brother-sister matings so far as possible. In general 2 females were bred with each male. The aim was to obtain 3 litters from each fertile female on the vegetarian diets and to use only members of second or third litters for continuing the lines. However, in some cases members of first litters were used for further breeding because no members of later litters were raised. Moreover, F_2 generation females were mated with F_1 males when F_2 males of the

[1] This study was aided by a grant from Swift & Co., Chicago.

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same strain seemed to be sterile, and finally F_2 Wistar females were mated with Sprague-Dawley males because all of the Wistar males that were reserved for breeding died. Litters of more than 8 young were reduced to 8 a few days after the litters were cast. After 3 litters were obtained on the vegetarian diet or the rats appeared to be sterile, they were fed an omnivorous diet (generally our basal omnivorous diet 1 ['47]), and its effect on the reproduction and growth of offspring was determined.

RESULTS

Data concerning the reproductive performance of the rats and the weights of their offspring at 42 days are presented in table 1. For comparison purposes, data are included in the table for rats fed the vegetarian diets, and for breeding rats fed our omnivorous diets. Data obtained on the 2 strains of

TABLE 1

Reproductive performance of rats on omnivorous and vegetarian diets and weights of offspring at 42 days.

GENERATION [1]	MALES MATED	Fertile	FEMALES MATED	Fertile	LITTERS CAST	INDIVIDUALS RAISED	MALES RAISED	Av. wt. at 42 days	FEMALES RAISED	Av. wt. at 42 days
	no.	*%*	*no.*	*%*	*no.*	*%*	*no.*	*gm*	*no.*	*gm*
				Rats on omnivorous diet						
F_0 to F_5	14	64	28	68	38	61	92	161	112	134
				Rats on vegetarian diets						
F_0	9	89	18	94	49	21	39	66	43	62
F_1	8	75	14	100	37	13	19	50	17	47
$F_1♂ \times F_2♀$	2	100 [2]	4	100	4	19	3	44	2	39
F_2	4	50	6	50	3	0				
				Rats on omnivorous diet after vegetarian diet						
F_0 to F_2 [3]	12	83	19	95	22	71	63	155	60	130

[1] F_0 generation omnivores means rats previously on a stock diet. F_0 generation vegetarians means offspring of rats fed omnivorous diets.

[2] Previously known to be fertile.

[3] Generations of vegetarians.

rats with the differently supplemented vegetarian diets are combined as no significant differences in the results were noted. As indicated, most of the rats on the vegetarian diets were fertile but they raised less than half the percentage of offspring raised by the rats fed our omnivorous diets, and these offspring of the vegetarians weighed less than half as much as those of the omnivores. Moreover, these young of the vegetarians weighed less in successive generations while those of the omnivores tended to be of equal weight. After the

TABLE 2

Season of the year in relation to type of diet, number of offspring born, and per cent of young raised.

DIET AND OFFSPRING BORN	SEASON OF YEAR			
	Dec.–Feb.	Mar.–April	May–Aug.	Sept.–Nov.
Omnivorous:				
Number born [1]	69	71	86	116
% raised	41	52	83	59
Vegetarian:				
Number born [1]	187	171	178	197
% raised	0	17	37	17
Omnivorous [2]*:*				
Number born [1]	47	57	46	22
% raised	74	56	85	73

[1] These are the number of rats born less the number sacrificed to reduce the size of litters of more than 8 new-born.

[2] After vegetarian diet.

vegetarians were fed one of our omnivorous diets, they raised a higher percentage of young than did the rats that were kept on our omnivorous diets, and their offspring immediately approached the weights of those of the omnivores. In fact, the male rat that became heaviest at 42 days (208 gm) was an offspring of ex-vegetarians.

Table 2 shows that the rats on the vegetarian diets raised no offspring in winter (3 winters included) and only 37% in summer. The omnivorous controls revealed a parallel but less extreme seasonal variation in the percentage of offspring

raised, but with respect to the vegetarians that were finally fed an omnivorous diet no significant seasonal influence on the percentage of offspring raised was observed.

Some of the offspring of the vegetarians were deformed at an early age but the deformities were evidently mainly due to the development of severe rickets. The inclusion of celery cabbage as a source of calcium (as well as of supplementary protein in the diet) did not prevent the development of severe rickets in some cases. At autopsy, enlarged joints and bowed fibula or tibia were found and the femurs of over half of the offspring of the vegetarians could not be removed without a separation of the head of the femur, due to poor calcification. This occurred even in offspring of the vegetarians that were over 200 days old and that had been fed our basal omnivorous diet (which included 3% bone meal) 30 days or more. Many of the offspring of the vegetarians were also more hyper-excitable and manifested more fear of being handled than did the offspring of rats on omnivorous diets, and the hyper-sensitivity did not decrease after the vegetarians were placed on an omnivorous diet. The sexual development of the vegetarians was retarded, and more so in the males than in the females. The feces of the vegetarians were often poorly formed.

DISCUSSION

The low percentage of offspring raised by the rats on the vegetarian diets was due to a high neonatal mortality and the apparent killing and eating of a large percentage of the young by the mothers. The killing of a pup by a vegetarian mother (by biting into the nape of the neck) was directly observed only once, but as many as 7 otherwise seemingly viable young were eaten by the vegetarian mothers during a single night. Occasionally, rats fed diets including meat also kill and eat their offspring (as observed) but the eating of a much larger percentage of offspring by our vegetarians was most likely due to the deficiencies in the vegetarian diets of suitable protein, calcium and other nutritional essentials. It is conceivable that the normal eating of the placentas after the

young are born whets the appetite of rats in need of such food and leads them to eat more than merely the placentas.

The failure of the vegetarians receiving celery cabbage to raise more offspring or heavier offspring than did the vegetarians receiving mainly lettuce as a fresh leafy vegetable supplement to the diet may be explainable by the relatively small amount of celery cabbage eaten. The rats ate much more lettuce than celery cabbage when both were supplied and they ate only slightly more celery cabbage when no lettuce was supplied. Some rats ate practically no celery cabbage, and the third generation of rats that were fed celery cabbage as the sole fresh leafy vegetable supplement to the diet still preferred lettuce when this was finally supplied. The lettuce fed by us consisted mainly of trimmings of head-lettuce (the green outer leaves and cuttings of the stems). The rats preferred this to leaf-lettuce, spinach, cabbage, celery, broccoli, cauliflower trimmings, carrot tops and beet tops. Variable amounts of greens other than lettuce were nevertheless usually also eaten when supplied with lettuce.

Considerable individual differences in the preferences or acceptances of the different items included in the basal part of the vegetarian self-selection diets were manifested by the rats, and changes in preferences occurred with ageing, pregnancy and in successive generations. The general order of preferences or acceptances of the different items was, however, approximately as follows: the germ part of kernels of corn, sunflower seeds, peanuts, green peas, the end of kernels of corn opposite the germ, defatted corn germ meal, barley, wheat, rolled oats, alfalfa leaf meal, soy beans, brewers' yeast, defatted wheat germ meal, salt (NaCl). The relatively low acceptance value of soy beans may help to explain why soy beans have not been used to any great extent even in China (according to Adolph, '44). Of special interest seemed to be the gnawing of the end of kernels of corn opposite the germ, mainly by young rats in the F_1 and F_2 generations. This end of the kernels of corn forms the surface of the ears and, as a result of pervaporation in the drying of the ears, the

surface evidently contains a concentration of water-soluble and presumably tasty components (Alexander, '33). The preference of the apparently more tasty part of the kernels of corn to the germ part suggests a finicky appetite. However, a sharp change to a preference of the germ part occurred in the females when they became pregnant. The general preference for the germ part of corn, sunflower seeds and peanuts to other items on the diet incidentally suggests that the fat or oil in these foods partly explains their appeal to the rats.

The period between weaning (at 35 days) and sexual maturity (as late as 150 days in some vegetarians) appeared to be the most difficult for the offspring of the vegetarians to bridge. About half of the males died during this period. The females fared better. Under more natural environmental conditions than those under which rats are kept in the laboratory they would evidently thrive better on an otherwise purely vegetarian diet as they would be able to resort to geophagy, osteophagy, the eating of insects and the eating of all dead rats. We removed dead rats, partly eaten rats and dying rats to prevent their being eaten. There was no evidence, however, of any tendency of adult vegetarians to resort to coprophagy as shown by rats on some vitamin deficient diets.

In contrast to the results of our study, Wu and Chen ('29) using a simple vegetarian diet supplemented alternately by colza and (Chinese) "small cabbage," found that their rats raised as high a percentage of offspring in the first 3 generations as rats fed their omnivorous stock diet. Their strain of rats may have been better adapted to living on a purely vegetarian diet than the strains we used, but it seems more likely that prolonged human experience of living on practically vegetarian diets in China has led to the cultivation of vegetables like colza and "small cabbage" which serve better as supplements to grains and legumes than leafy vegetables commonly raised in the United States. Moreover, the soil and agricultural methods used in China may serve to produce foods (grains and legumes as well as leafy vegetables) of

higher biological value than similar foods grown in the United States. Wu and his associates eventually raised 25 (or more) generations of rats with their vegetarian diet but the growth of the rats was subnormal and, after the first generation, 70% developed cataracts (Chen, Chang and Luo, '41). A purely vegetarian diet satisfactory for albino rats therefore still remains to be found.

SUMMARY

Rats on vegetarian self-selection diets including corn (whole kernels), wheat (whole grains), pearled barley, rolled oats, sunflower seeds, peanuts, soy beans, green peas, corn germ meal, wheat germ meal, brewers' yeast, alfalfa leaf meal, salt (NaCl), lettuce and celery cabbage were found to be generally fertile but raised less than 25% of their offspring. They raised none in winter. The growth of these young was also subnormal and severe rickets commonly developed.

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RELATION OF CARBOHYDRATE TO INTESTINAL SYNTHESIS OF BIOTIN AND HATCHABILITY IN MATURE FOWL [1,2]

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FIVE FIGURES

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It is well-recognized that changes in the kind of dietary carbohydrate may be reflected in the amount of vitamins synthesized by intestinal microorganisms (Guerrant, Dutcher and Tomey, '35; Morgan, Cook and Davison, '38; Mannering, Orsini and Elvehjem, '44; Najjar and Barret, '45; and Teply, Krehl and Elvehjem, '47). Evidence has been presented which shows that biotin is synthesized in animals (McElroy and Jukes, '40; Wegner et al., '41; Nielsen et al., '42; Mitchell and Isbell, '42) and in humans (Gardner et al., '43, '45, '46; Oppel, '42); it is synthesized by the bacteria of the intestinal tract (Landy et al., '42; Thompson, '42; Burkholder and McVeigh, '42).

The laying hen appeared to be a desirable experimental animal for studying intestinal synthesis since the vitamin content of the egg could be determined and would be a measure of the biotin available for metabolism. The use of the egg

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as a measure of intestinal synthesis eliminates certain difficulties associated with collecting and assaying urine and feces in the chicken. Cravens, Sebesta, Halpin and Hart ('42) reported that biotin was necessary for normal embryonic development in the domestic fowl.

The present investigation was initiated to determine the effect of different carbohydrates on intestinal synthesis of biotin in the mature fowl. Sucrose, dextrin, and combinations of sucrose with lactose or dried whey were tested. Egg production, hatchability of fertile eggs, and biotin content of the eggs were the criteria used in evaluating the extent of intestinal synthesis and absorption of biotin.

MATERIALS AND METHODS

Twenty-four Single Comb White Leghorn pullets were placed in individual laying cages with raised screen bottoms, after being reared on the Experiment Station range. All birds were artificially inseminated weekly with mixed semen from New Hampshire cockerels. All eggs laid were marked with the hen's number and the date and settings were made weekly. The eggs were candled on the sixth and seventeenth days of incubation, at which times the infertile eggs and dead embryos were removed. All eggs which were removed on candling, and also those which failed to hatch, were broken and the age of the embryo at the time of death was estimated. Any gross abnormalities were recorded.

The pullets were fed an all-mash laying ration for 4 weeks prior to the start of the experiment, during which period data were collected on egg production and hatchability. At the end of this pre-experimental period the 24 birds were divided into 6 groups of 4 pullets each. At the end of the ninth week of the experimental period, the pullets which had been receiving diets B33 and B35 were placed on the practical all-mash diet and received the latter during the final week of the experiment. This change was necessitated by the poor condition of the birds at the end of the ninth week.

The materials used in this study were sucrose, dextrin (prepared by autoclaving moist cornstarch 2 hours with subsequent drying and grinding), lactose and dried whey. The composition[4] of the diets is shown in table 1. Crystalline B-vitamins were added in amounts which were thought to meet

TABLE 1

Composition of diets.

	B31	B32	B33	B34	B35
Sucrose — %	63		48	63	38
Dextrin — %		63			
Purified casein — %	18	18	18	18	18
Gelatin — %	5	5	5	5	5
Salts IV — %	5	5	5	5	5
Liver fraction "L" — %	4	4	4	4	4
Fish oil (3000 A) (400 D) — %	2	2	2	2	2
Soybean oil — %	3	3	3	3	3
Lactose — %			15		
Dried whey — %					25
Choline — %	0.2	0.2	0.2	0.2	0.2
Oyster shell			Ad libitum		
All vitamins listed below added as indicated in mg per kg of ration					
Biotin				0.2	
Thiamine HCl	4	4	4	4	4
Riboflavin	6	6	6	6	6
Ca pantothenate	15	15	15	15	15
Niacin	100	100	100	100	100
2-methyl-1,4-naphthoquinone	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	4	4	4	4	4
Alpha tocopherol	3	3	3	3	3

the requirements of the pullets with the exception of biotin and pteroylglutamic acid. Liver fraction "L" was used as a source of the latter vitamin. Biotin was omitted from all diets except B34 since this vitamin was the one under study. A practical all-mash diet was used as a positive control in addition to diet B34. Oyster shell and tap water were supplied ad libitum.

[4] We are indebted to the Western Condensing Co., for the lactose and dried whey; to Wilson Laboratories, Chicago, Illinois, for the liver fraction "L"; to Merck and Co., Rahway, N. J., for the biotin.

Eggs which were to be assayed for biotin were set aside on the last 2 days of each week. The microbiological method of Luckey, Moore and Elvehjem ('46), with slight modification, was used. The egg whites and egg yolks were separated and those from each group of birds were pooled for assay. After thorough mixing, approximately 10 gm were weighed out. An equal number of milliliters of 4N sulfuric acid was added and the sample autoclaved for 2 hours at 15 pounds pressure. In preliminary assays on eggs it was determined that hydrolysis with 4N, 8N or 12N sulfuric acid, as outlined above, gave approximately the same results. The 4N acid was selected since the sample would contain sodium sulfate on neutralization. After autoclaving, the pH was adjusted to 4–5 with 4N sodium hydroxide and the samples were filtered through Whatman no. 1 filter paper. The filtrate was neutralized to pH 6.8–7.0 and preserved under toluene in the refrigerator. Dilutions were made as needed. The organism used for the assay was Streptococcus faecalis R. The extent of growth of the organism was measured by turbidity using the Evelyn colorimeter. Satisfactory recoveries of biotin were obtained when known amounts of the vitamin were added to the samples. The biotin content of the egg whites and yolks is expressed in millimicrograms per gram on a fresh weight basis.

EXPERIMENTAL RESULTS

Egg production. The production of pullets fed the various diets is shown in figure 1. The egg production of pullets fed diets B31 and B32 and those fed the practical all-mash diet followed the same general trend throughout the 10-week period although a slight drop in the egg production of pullets fed sucrose, diet B31, may be noted during the last week of the experiment. The egg production of pullets fed sucrose plus biotin, diet B34, was somewhat erratic. The fluctuations in this case were traceable to 1 pullet and are more apparent due to the small number of pullets in the group; this pullet would lay a clutch of eggs and go out of production, then come back in production and repeat the same procedure.

The rather high levels of lactose and dried whey appeared to cause a decrease in egg production. The production of pullets fed diets B33, lactose, and B35, dried whey, decreased during the third and fourth weeks of the experimental period and remained at a fairly low level for the rest of the 10-week period. This low production was unfortunate because an insufficient number of eggs were laid to obtain a measure of

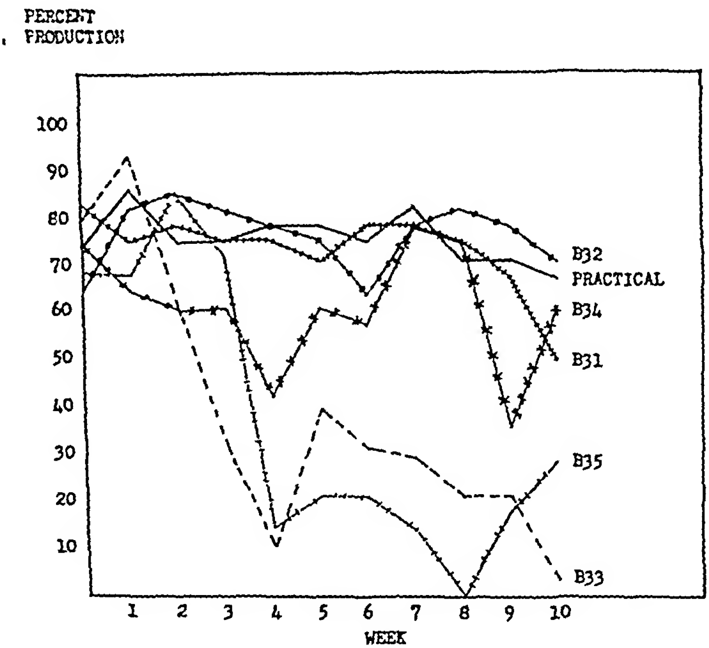

Fig. 1 Effect of diet on egg production: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

hatchability and for biotin assay in the case of pullets fed dried whey (diet B35).

Hatchability. The per cent hatchability of fertile eggs is shown in figure 2. Hatchability of eggs from pullets fed diet B31, sucrose, decreased to zero by the third week and remained at this level. Hatchability of eggs from pullets fed diet B32, dextrin, ranged from 60 to 85% during the 10-week period. This was somewhat lower than that of the positive control

groups which were fed diet B34 and the practical all-mash diet. Hatchability of eggs from pullets fed diet B34, sucrose plus biotin, was approximately the same as that of those fed the practical all-mash diet. The results with respect to hatchability obtained with diets B33, lactose, and B35, dried whey, were somewhat inconclusive after the third and fourth weeks due to the poor egg production which resulted from the

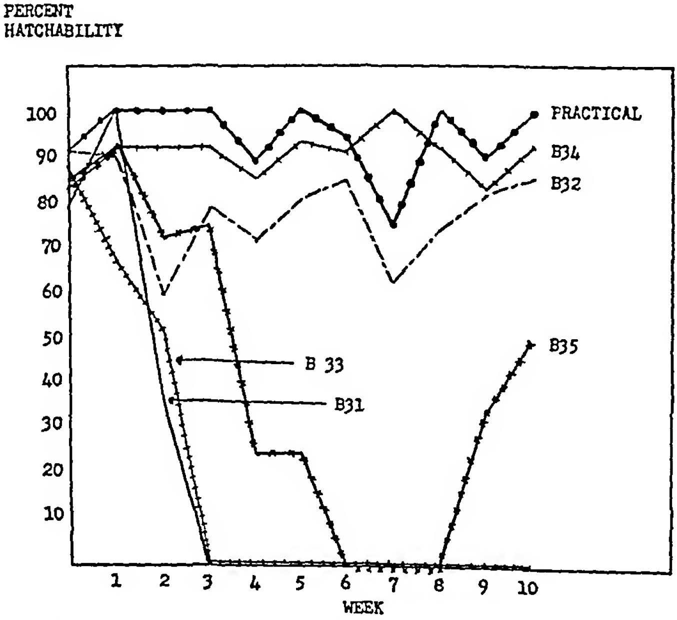

Fig. 2 Effect of diets on hatchability: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

amounts of these materials used in this study. This was discussed above.

Biotin content of egg white and yolks. At the beginning of the experiment the biotin content of the egg whites (fig. 3) varied from 65 to 105 mμg per gram expressed on a fresh weight basis. The biotin content of egg whites from hens fed diet B31 decreased from 65 to 17 mμg per gram during the first week and the presence of the vitamin was not detected in egg whites from these birds after this time. The level of biotin in

the whites of eggs from pullets fed diet B32 decreased from 88 to 22 mμg per gram during the first week and remained at the latter figure until the end of the third week; during the fourth week there was a further decrease from 22 to about 4–5 mμg of biotin per gram and it remained at this level through the eighth week.

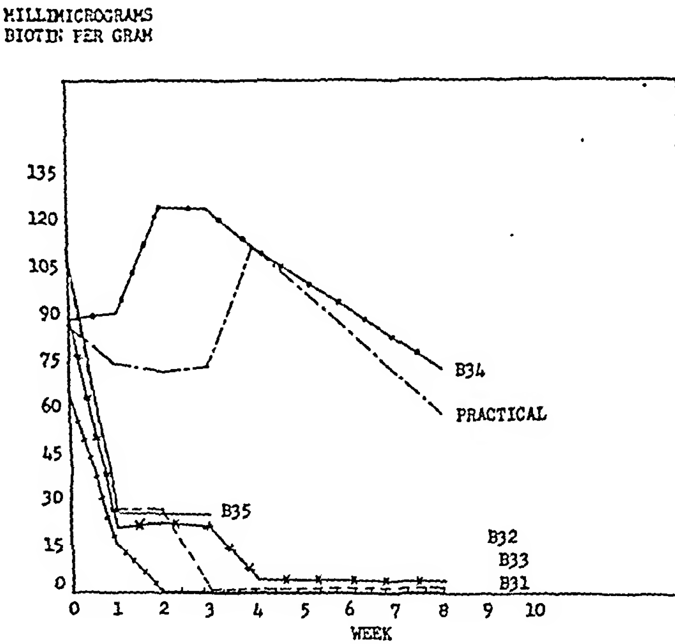

Fig. 3 Effect of diet on biotin content of egg white: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

The biotin content of egg whites from pullets fed diet B33 decreased from 105 to 27 mμg per gram during the first week and remained at the latter figure throughout the second week. At the end of the third week and thereafter, biotin could not be detected in egg whites from pullets fed diet B33. There was a decrease in the biotin content of egg whites from pullets fed diet B35 from 105 to 26 mμg of biotin per gram during the first week and it remained at the latter figure until the end of the third week. No eggs were analyzed from

pullets fed diet B35 after the third week due to the poor egg production of the birds. The feeding of 200 μg of biotin with sucrose, diet B34, was sufficient to maintain a slightly higher level of the vitamin in egg whites than was maintained in egg whites of pullets fed the practical all-mash diet (fig. 3).

The egg yolks from the pullets used in this study contained approximately 500 mμg of biotin per gram at the start of the

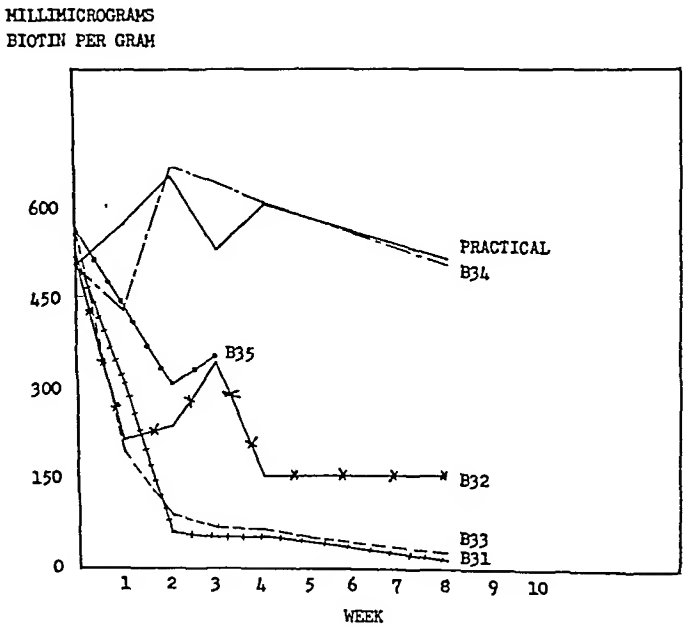

Fig. 4 Effect of diet on biotin content of egg yolk: diet B31, sucrose; B32, dextrin; B33, lactose; B34, biotin; B35, dried whey.

experiment (fig. 4). The biotin content of the yolks from pullets fed diets B31, sucrose, and B33, lactose, decreased from about 500 to 50–70 mμg of biotin per gram during the first 2 weeks of the experiment. The egg yolks from pullets fed diet B32, dextrin, showed a decrease of from 500 to approximately 150 mμg of the vitamin per gram and remained at the lower level. The biotin content of egg yolks from pullets fed diet B35 followed the same general trend as those

TABLE 2

Effect of diet on incidence of syndactyly and other skeletal deformities.

HEN	DIET	NO. OF FERTILE EGGS	EMBRYOS DYING		NUMBER OF EMBRYOS SHOWING			SYN-DACTYLY
			Before 8 days	After 8 days	Beak par-rot	Crooked tibia	Short twisted tarso meta-tarsus	
1	B-31	49	19	18	8	7	7	7
2		13	3	7	3	6	6	6
3		16	14	0	0	0	0	0
4		35	18	14	8	2	2	5
	Total	113	54	39	19	15	15	18
5	B-32	40	2	2	0	0	0	0
6		48	2	6	0	0	0	0
7		49	2	4	0	0	0	0
8		43	7	9	0	0	0	0
	Total	180	13	21	0	0	0	0
9	B-33	28	9	11	3	5	5	4
10		13	7	1	0	0	0	0
11		11	1	4	4	4	4	3
12		9	1	7	1	2	2	4
	Total	61	18	23	8	11	11	11
13	B-34	37	2	3	0	0	0	0
14		42	1	0	0	0	0	0
15		33	0	4	0	0	0	0
16		6	0	0	0	0	0	0
	Total	118	3	7	0	0	0	0
17	B-35	26	10	4	0	2	2	1
18		15	1	0	0	0	0	0
19		6	0	0	0	0	0	0
20		8	1	1	0	0	0	0
	Total	55	12	5	0	2	2	1
21	Practical	42	1	2	0	0	0	0
22	Control	39	0	1	0	0	0	0
23	Diet	49	0	2	0	0	0	0
24		37	0	3	0	0	0	0
	Total	177	1	8	0	0	0	0

from pullets fed diet B32. Diet B34 and the practical all-mash diet supported about the same concentration of the vitamin in the egg yolks.

Embryonic mortality. The classification of the embryos that died during the incubation period is shown in table 2. There was a high incidence of micromelia and syndactyly in dead embryos from pullets fed diet B31. The micromelic embryos

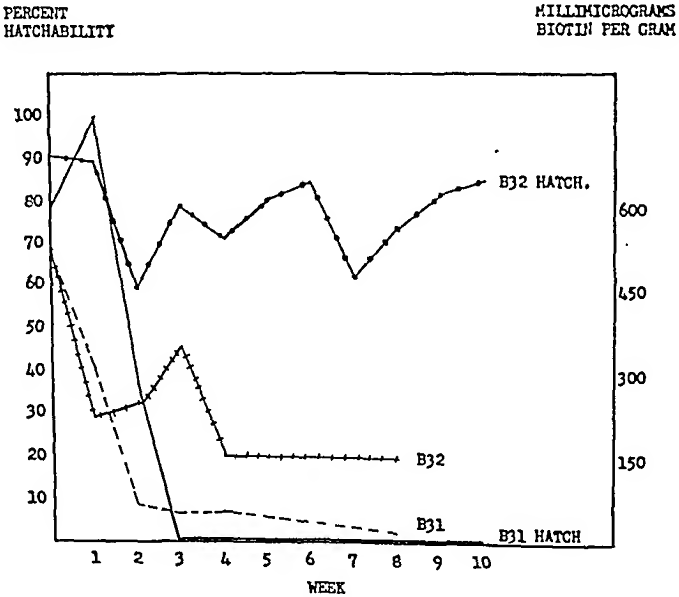

Fig. 5 Relation of carbohydrate to biotin content of egg yolk and hatchability: diet B31, sucrose; B32, dextrin.

were characterized by a parrot beak which was generally associated with a crooked tibia and a short twisted tarsometatarsus. Syndactyly and micromelia were not observed in dead embryos from pullets fed diet B32, diet B34 or from those fed the practical all-mash diet. The incidence of micromelia and syndactyly was high in the dead embryos from pullets fed diet B33 and was low in those from pullets fed diet B35, although these results are inconclusive due to the poor egg production of the birds.

DISCUSSION OF RESULTS

It may be noted from figures 2, 3, 4 and 5 that dextrin (diet B32) maintains a higher level of biotin in the egg yolk than does sucrose (diet B31) and that this is associated with a much higher per cent hatchability. The biotin content of egg yolks from pullets fed diet B31 decreased from about 500 to 50 mμg of biotin per gram during the first 3 weeks and had decreased to about 16 mμg of the vitamin per gram by the end of the eighth week. The decrease in the per cent hatchability of eggs from pullets fed diet B31 paralleled the decrease in biotin content of the yolk (fig. 5). The per cent hatchability was 79 at the start of the experiment. This increased to 100% for some unknown reason during the first week and decreased to zero by the end of the third week. It is apparent therefore that there was an insufficient amount of biotin to support embryonic development in the eggs from pullets fed diet B31. Thus it is indicated that eggs containing 50 mμg of biotin per gram of yolk and an undetectable amount in the white will not support normal embryonic development. In the case of pullets fed diet B32 there was a decrease in the biotin content of the yolk to about 150 mμg by the end of the fourth week and this amount of biotin was found in the yolks at the end of the eighth week. Hatchability of eggs from pullets fed diet B32 was 91% at the start of the experiment and varied from 60 to 85% in the course of the 10-week period. It is realized that the per cent hatchability of eggs from pullets fed diet B32 is lower than that of pullets fed diet B34 and also lower than that of pullets fed the practical all-mash diet. Embryonic abnormalities which appeared to be identical with those previously described by Cravens et al. ('44), as occurring in eggs from hens fed a low-biotin diet, were noted in eggs from pullets fed diet B31 but did not appear in eggs of pullets fed diet B32 (table 2). From these data it is indicated that eggs which contain 150 mμg of biotin per gram of yolk on a fresh weight basis will support embryonic development even though hatchability may be somewhat reduced.

Supplementation of the sucrose diet with biotin (diet B34) resulted in a per cent hatchability and biotin content of the eggs comparable to those obtained by feeding a practical all-mash diet (figs. 2, 3 and 4). These data indicate that the deficiency which existed in the pullets fed diet B31 was a biotin deficiency.

The results obtained with dextrin (diet B32) as compared to those obtained with sucrose (diet B31) suggest that there is a marked synthesis of biotin in the intestinal tract. Further, there is good evidence that the biotin synthesized in the intestinal tract of the pullets fed dextrin was absorbed and deposited in the egg. This latter fact was established through biotin analyses of the eggs and by the fact that the biotin in the eggs was available to the embryo as evidenced by the per cent hatchability.

This is in agreement with the work of Mannering et al. ('44) in which it was reported that dextrin favored the synthesis of riboflavin in the rat, and also with the work of Teply et al. ('47) in which it was reported that dextrin diets produced the greatest synthesis of niacin and folic acid in the rat.

The data presented in this paper indicate that the laying hen is a desirable animal for studying intestinal synthesis since a determination of vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult with other animals.

The studies reported herein shed no light on the important problem of where synthesis and absorption take place in the intestinal tract. Studies by Johansson et al. ('47) which were conducted on the birds used in the present investigation indicate that there was a marked difference in the intestinal flora of birds fed the various diets. Dextrin appeared to favor a biotin-synthesizing organism while sucrose favored a biotin-utilizing organism.

The biotin content of the eggs did not appear to be related to the egg production of the hens in the cases of pullets fed

diets B31 (sucrose) or B32 (dextrin). This is in agreement with the work of Cravens et al. ('42).

Results obtained by replacing a part of the sucrose with lactose (diet B33) or with dried whey (diet B35) are somewhat inconclusive due to the poor egg production of the pullets fed these diets. It is probably true that the level of lactose and dried whey used in these diets was too high for best results and suggests that the laying pullet may not tolerate lactose in quantities equal to that of the rat (Geyer et al., '46). Lactose, in the quantity used in diet B33, did not appear to favor the intestinal synthesis of biotin (figs. 2, 3 and 4). The biotin content and per cent hatchability of eggs from pullets fed diet B33 (lactose) followed the same general trend as those of pullets fed diet B31 (sucrose). Embryonic abnormalities, which were associated with a low biotin diet, were also observed in embryos from pullets fed diet B33 (table 2).

The results obtained with diet B35 (dried whey) were quite inconclusive after the third or fourth week due to the poor egg production of the pullets fed this diet (fig. 1). There was a higher level of biotin in the egg yolks and whites and a higher per cent hatchability of eggs from pullets fed diet B35 during the first 3 weeks than was noted in eggs from pullets fed diets B31 and B33. This may have been traceable to the fact that 25% dried whey contributed approximately 40 µg of biotin per kilogram of diet. A small number of embryos exhibiting symptoms of a biotin deficiency were noted in the eggs of 1 pullet fed this diet (table 2).

The term "micromelia" is used in contrast to the term "chondrodystrophy" in referring to the characteristic embryonic abnormality associated with a deficiency of biotin in the diet of the mother hen. It is recognized that the term "chondrodystrophy" designates a certain histopathologic picture. It should be pointed out, however, that from a gross macroscopic study the micromelia embryos recorded in table 2 of the present report and described by Cravens et al. ('44) are indistinguishable from the chondrodystrophy which has been shown to have a hereditary basis (Lamoreaux, '42).

The occurrence of micromelia and syndactyly in embryos from pullets fed diet B31 extends and confirms the previous work of Cravens et al. ('44). It may be noted from table 2 that the characteristics which describe micromelia: parrot beak, crooked tibia and short-twisted tarsometatarsus appeared to occur in about the same frequency as syndactyly. This indicates that syndactyly is a definite symptom of biotin deficiency in the developing chick embryo as was also observed by Cravens et al. ('44). Micromelia and syndactyly were observed among embryos which lived beyond the eighth day of incubation from pullets fed diets B31 and B33. The fact that eggs from these birds were low in biotin has been discussed earlier. The incidence of micromelia and syndactyly appeared to be fairly well distributed among the individual pullets fed diets B31 and B33 (table 2).

SUMMARY

Evidence is presented to show that dextrin favors the intestinal synthesis of biotin in the mature fowl and sucrose does not promote the synthesis of this vitamin. The results obtained with lactose and dried whey indicate that lactose does not stimulate the synthesis of biotin in the intestinal tract of the laying pullet.

The laying hen is a desirable animal for studying intestinal synthesis. A determination of the vitamin content of the egg offers a method of evaluating the extent of synthesis and subsequent absorption of vitamins from the intestinal tract which is difficult in other animals.

There is a failure of embryonic development when the egg yolk contains as little as 50 mμg of biotin per gram on a fresh weight basis. Eggs which contain 150 mμg of biotin per gram will support normal embryonic development.

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HUMAN UTILIZATION OF ASCORBIC ACID FROM MUSTARD GREENS

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TWO FIGURES

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Previous investigations have shown that, for many families, cooked vegetables are the main dietary source of ascorbic acid (Grigsby et al., '42; Bryson et al., '42; Coco et al., '43; Whitehead, '47). In the far South cooked greens, repeatedly shown by chemical determination to be good sources of vitamin C, are commonly used. A study of the availability for human subjects of vitamin C from such sources is therefore of practical value to the nutritionist.

A number of studies have been reported which pertain to the utilization of dietary ascorbic acid as compared to that of the crystalline vitamin. The levels at which the vitamin has been fed, the number of subjects and the duration of the test periods have varied widely. Some workers have based conclusions on urinary excretion of the ascorbic acid, while others have used plasma or whole blood levels, and a few have used both plasma or whole blood levels and urinary excretion.

After a study of the urinary excretion of ascorbic acid following the administration of comparable quantities of this vitamin as orange juice and in the pure crystalline form, Hawley, Stephens and Anderson ('36) concluded that the vitamin C of orange juice was as well utilized as the pure compound.

Baked potato was found by Clayton and Folsom ('40) to produce as high plasma ascorbic acid values as did the crystalline vitamin. Two-thirds of the dietary ascorbic acid was supplied by this vegetable.

Todhunter and Fatzer ('40) compared the utilization of ascorbic acid from red raspberries with that of the crystalline vitamin. Blood plasma ascorbic acid levels and urinary excretion of ascorbic acid were similar for the same subject, regardless of the source of the vitamin.

In 1942 Todhunter, Robbins and McIntosh reported a study of the rate of increase of blood plasma ascorbic acid after ingestion of 50 mg of ascorbic acid in the crystalline form, and after consumption of equivalent amounts in cauliflower, orange sections, orange juice or strawberries. Orange juice and orange sections produced the maximum rise in plasma level as quickly as the pure substance, while strawberries required slightly longer, and cauliflower the longest time of all the foods tested.

Using the ability of the supplement to maintain tissue saturation and to support plasma ascorbic acid levels as the criteria for judging availability, Clayton and Borden ('43) concluded that both canned tomato juice and raw cabbage produced as good results as the vitamin in tablet form.

Hartzler ('45) found no significant differences in the availability of the ascorbic acid of papayas or guava juice as compared with synthetic ascorbic acid. The criteria used for comparison of availability were the daily urinary excretion and plasma ascorbic acid levels determined once each week.

Einbecker et al. ('47) found that increases in plasma ascorbic acid produced by a test meal including frozen strawberries were comparable to those obtained when crystalline vitamin C was taken in amounts equivalent to the reduced ascorbic acid in the strawberries. Increases obtained when crystalline ascorbic acid was taken in amounts equivalent to the apparent total ascorbic acid of the strawberries were greater than those obtained after the ingestion of strawberries.

Elliott and Schuck ('47) reported approximately equal utilization of ascorbic acid from grapefruit and from the crystalline vitamin. The criteria upon which this conclusion was based were urinary excretion of ascorbic acid by 9 subjects during two 3-day test periods and whole blood ascorbic acid levels of 3 subjects at the end of each test period.

EXPERIMENTAL PROCEDURES

Mustard greens were chosen as the green leafy vegetable for this experiment because they are widely used in the South and a good supply was available at a convenient time.

The daily basal diet consisted of 1 egg, 1 pint of pasteurized milk, and 1 serving of each of the following: a dried fruit, a canned fruit, thoroughly cooked meat, canned snap beans, and a cooked fresh vegetable. Carrots, eggplant, potatoes, and mature dry onions were the vegetables used. The eggplant was sautéed, while the other fresh vegetables were boiled in a large amount of water to reduce the ascorbic acid content. The potatoes were always mashed. Whole grain and enriched cereals, bread, cake, cookies, and baked beans were used *ad libitum*.

During the periods when the mustard greens were eaten, the greens were substituted for the canned snap beans in the noon meal. During the periods when the synthetic vitamin was used, the ascorbic acid supplement was also given at the noon meal. Since the diet was believed to be adequate in all respects except ascorbic acid, no other vitamin supplements were given. The mustard greens were blanched and frozen before the beginning of each experiment to insure a regular supply. A pressure saucepan was used for cooking as previous experience (Hollinger, '44; Hollinger and Colvin, '45) had shown that this method of cooking results in maximum retention of vitamin C. The greens were seasoned with sufficient salt and bacon fat before cooking to make them palatable.

In 1945, 5 young women served as subjects. The plan of the experimental periods is given below.

Experimental periods — 1945

Period I, 14 days. Basal diet containing 13 mg of reduced ascorbic acid.

Period II, 21 days. Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap beans. The average ascorbic acid content of this diet was 57 mg, of which 49 mg were supplied by the greens.

Period III, 14 days. Basal diet as in Period I with supplement of ascorbic acid in water solution to give same average total intake as in Period II.

Immediately after breakfast blood samples were drawn by venous puncture on 5 consecutive days each week. Analyses were made immediately by the Mindlin and Butler ('38) macro method. The method was followed in detail except that no potassium cyanide was used.

All foods containing ascorbic acid were eaten in constant weighed amounts and a determination of the reduced ascorbic acid was made daily on a composite sample representing one-fourth of the intake. Determination of ascorbic acid in the food was made by the dye titration method.

In 1946 the plan of the experiment was altered in order to provide for a period of high ascorbic acid intake immediately preceding the beginning of the experimental period and again following the period when mustard greens were eaten. The plan of the experimental period is given below. This plan insured a more uniform state of nutrition among the subjects than is probable when no preparation of the subjects is carried out.

Experimental periods — 1946

Period I, 15 days. Uncontrolled diet. One hundred milligrams ascorbic acid daily in tablet form.

Period II, 14 days. Basal diet containing an average of 15 mg of ascorbic acid.

Period III, 15 days. Basal diet supplemented by 125 gm of mustard greens at the noon meal in place of canned snap

beans. The ascorbic acid content of this diet was 57 mg, of which 49 were supplied by the cooked greens.

Period IV, 15 days. Same as Period I.

Period V, 14 days. Same as Period II.

Period VI, 15 days. Basal diet supplemented by sufficient ascorbic acid in solution to bring the average total intake of the vitamin to that in Period III.

Blood samples were drawn immediately after breakfast 4 times each week. The samples were taken on Monday, Tuesday, Thursday and Friday in order to compare values on consecutive days. Eight samples from each subject were analyzed during the depletion periods (Periods II and IV) and 10 during the periods when the greens and the ascorbic acid were given (Periods III and VI).

Three young men and 4 young women served as subjects for this portion of the study. Three of the young women (subjects J.G., L.J., and V.W.) had served as subjects in 1945.

RESULTS

In 1945 the average plasma ascorbic acid values for 3 subjects were almost the same on both regimes. For 1 subject the average plasma ascorbic acid value was 14% higher on the ascorbic acid regime than on the mustard green regime, while in another the crystalline vitamin produced an average plasma ascorbic acid value 33% higher than that produced by mustard greens (table 1). In 1946, 6 subjects showed slight increases in plasma ascorbic acid values during the mustard green regime when these values were compared to the average for the 2 days just previous. One subject had the same average plasma value (table 2). During the period when ascorbic acid was taken, 4 subjects showed gains while the remaining 3 showed slight losses. Subject V.W. showed considerably greater gain on crystalline ascorbic acid than on mustard greens. It should be noted that this subject had served in the 1945 study and at that time also attained higher plasma ascorbic acid values when receiving the pure vitamin. Subjects

J.G. and L.J. who also had served in the 1945 study showed in both experiments approximately the same plasma ascorbic acid values on the ascorbic acid regime as on the mustard greens diet.

TABLE 1

Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1945.

SUBJECT	SEX AND WT. IN KG	PERIOD I BASAL DIET [1]	PERIOD II MUSTARD GREENS [2]	PERIOD III ASCORBIC ACID [3]
D. C.	F. 57	0.50	0.46	0.48
J. G.	F. 68	0.67	0.53	0.53
L. J.	F. 66	0.30	0.48	0.51
H. L.	F. 57	0.45	0.58	0.66
V. W.	F. 50	0.58	0.58	0.76
Average		0.50	0.53	0.59

[1] Average of 2 days preceding mustard green period.
[2] Average of 15 determinations during 3 weeks.
[3] Average of 10 determinations during 2 weeks.

TABLE 2

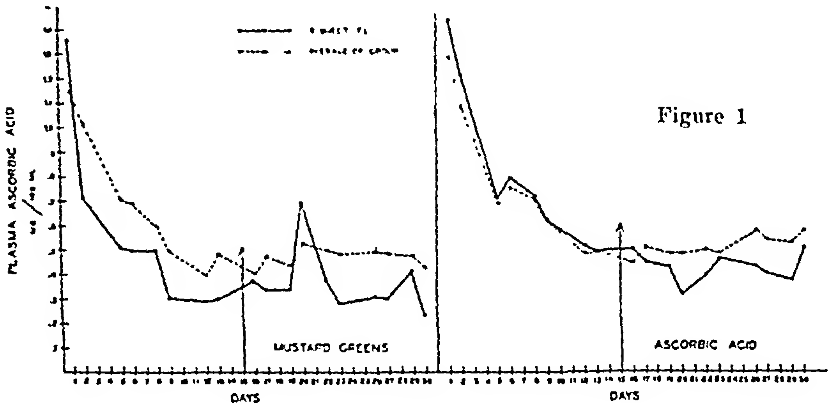
Average plasma ascorbic acid values produced by mustard greens and by crystalline ascorbic acid (in milligrams per cent) 1946.

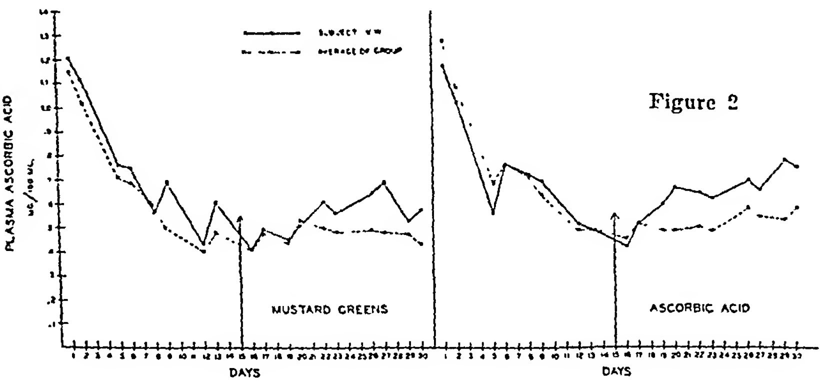
SUBJECT	SEX AND WT. IN KG	AT END OF PERIOD II [1]	MUSTARD GREENS [2]	AT END OF PERIOD V [1]	CRYSTALLINE ASCORBIC ACID [2]
J. B.	M. 60	0.39	0.46	0.52	0.51
J. G.	F. 68	0.47	0.52	0.48	0.51
V. H.	F. 60	0.56	0.57	0.54	0.61
L. J.	F. 66	0.45	0.45	0.50	0.49
F. L.	M. 72	0.29	0.36	0.50	0.43
J. W.	M. 76	0.36	0.39	0.36	0.39
V. W.	F. 50	0.52	0.54	0.50	0.63
Average		0.44	0.47	0.48	0.51

[1] Average of plasma values for last 2 days of period.
[2] Average of plasma values (10 determinations during 2 weeks).

Higher plasma values were maintained at all times by the smaller subjects while the larger subjects showed a more rapid decline on the basal diet and a very slow decline on both the mustard green and the crystalline ascorbic acid regime.

This suggests that the average daily intake of ascorbic acid, 59 mg, was inadequate for the larger individuals. Typical curves are shown in figures 1 and 2. Plasma values for the men showed fluctuations similar to those for the women.





Figs. 1 and 2 Comparison of plasma ascorbic acid values of subjects F.L. and V.W. and average plasma ascorbic acid values of 7 individuals — 1946.

CONCLUSION

It is concluded that some individuals utilize the ascorbic acid from cooked mustard greens just as well as the synthetic vitamin. Other persons fail to show as high plasma ascorbic acid values when mustard greens are the main source of the vitamin as they do when the crystalline vitamin is given.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

III. LOW PROTEIN INTAKE [1]

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Two recent publications in this journal by Forbes, Swift and associates ('46a, '46b) demonstrated that liberal amounts of fat in the diet of the growing rat resulted in a superior energy utilization of the diet as a whole. Increased body weight, comprising greater gains of fat and energy, and a decrease in the heat production of growing rats resulted as the fat content of isocaloric diets was increased from 2 to 30%. In the preparation of the diets used in these experiments an effort was made to furnish all nutrients in optimum quantities for the most efficient utilization of the food energy, including an excellent quality protein mixture furnishing 22% protein in the diet.

In view of the shortages of foodstuffs current in many areas of the world, especially of fat and protein, it seemed of further interest to investigate the effect of decreasing the dietary protein in similar high and low fat diets on the resulting energy utilization. It was decided, therefore, to follow the plan of the earlier experiments, namely, to use diets containing 2 and 30% of fat, but with a protein content of 7% in the low fat diet and an equivalent amount in the high fat diet.

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EXPERIMENTAL

The experimental subjects were 12 pairs of litter-mate male albino rats of the Wistar strain selected immediately after weaning. Each rat of a pair was fed isocaloric amounts of one of the 2 diets for a period of 70 days, using the paired-feeding technique. Both rations supplied the same amount of protein and vitamin supplements, and differed inversely in their fat and carbohydrate contents.

The subjects were allowed normal cage activity. The heat production as determined by the body balance method of this laboratory (Swift et al., '34) provided a single measurement for the entire 70-day period through the subtraction of the energy of the excreta and of the body gain from the gross energy of the food.

Daily feces and urine collections were made according to the method referred to above and all energy values of the diets, bodies, and excreta were determined by the bomb calorimeter. Nitrogen and crude fat determinations were made according to the Kjeldahl and Soxhlet extraction procedures, respectively.

The 2 diets, prepared from purified foodstuffs in the manner described earlier, were analyzed before the start of the experiment and stored in the refrigerator. Tables 1 and 2 present the composition of the diets and the quantities of major nutrients supplied.

The average weekly live weights for the 12 rats on each of the 2 diets are shown in table 3. It is apparent that the animals on the high fat diet gained more weight than the low fat group as the experiment progressed. The statistical odds that this difference in weight gain is significant are 50:1. The difference in live weight is further emphasized, however, by the method of food assignment used which has the effect of placing at a disadvantage those animals that increase more rapidly in weight since a larger proportion of the food intake is then required for maintenance and less is available for growth.

The possibility that this increased weight gain could be in the form of water is contraindicated by the data on the body

TABLE 1

General composition of diets.

COMPONENTS	DIET 1	DIET 2
	%	%
Protein containing mixture[1]	11.00	15.63
Carbohydrate mixture[2]	83.00	50.37
Corn oil[3]	2.00	2.00
Lard[4]	0.00	28.00
Salt mixture[5]	4.00	4.00
Cal. per gm	3.869	5.498
Isocaloric factors	1.000	0.704

[1] Casein 50%, skim milk powder 25%, irradiated yeast 25%. Mixture contained 60.5% protein (N × 6.25) and 4.71 cal. per gm.

[2] Corn starch 34%, sucrose 33%, dextrin 12%, and dextrose (cerelose) 21%. Mixture contained 3.75 cal. per gm.

[3] Mazola. Contained 9.52 cal. per gm.

[4] Contained 9.33 cal. per gm.

[5] U.S.P. XII. no. 2.

TABLE 2

Vitamins added per 1 g of diet 1 and to isocaloric quantities of diet 2.

VITAMINS		VITAMINS	
	mg		mg
Carotene	40	Choline chloride	2000
Thiamine hydrochloride	20	Alpha tocopherol	200
Riboflavin	20	P-aminobenzoic acid	200
Pyridoxine hydrochloride	20	2 methyl 1, 4 naphthoquinone	6
Niacin	20		
Calcium pantothenate	100	Inositol	2000

TABLE 3

Average live weights of rats during 10 weeks on isocaloric quantities of diets containing 2% and 30% of fat.

FAT CONTENT OF DIETS	INITIAL BODY WEIGHT	WEEK NUMBER 1	2	3	4	5	6	7	8	9	10
%	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm	gm
2	54	58	70	81	89	97	105	113	123	129	135
30	54	58	71	83	91	99	107	116	126	133	140

gain of fat which are summarized in table 4. The rats on the high fat diet gained, on an average, 14% more energy in the form of fat than the group on the low fat diet. The statistical odds that this difference is not due to chance are 25:1.

The average gain of ether extract in the rats on the 2% fat diet was 19.4 gm, while their average intake was only 11.5 gm.

TABLE 4

Average food consumption during 70 days; and initial and final nitrogen and fat contents of rat bodies.

FAT CONTENT OF DIETS	FOOD CONSUMPTION	NITROGEN INTAKE	BODY NITROGEN		BODY FAT	
			Initial	Final	Initial	Final
%	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
2	576.4	6.48	1.30	3.85	4.13	19.36
30	405.8	6.31	1.30	3.86	4.13	21.67

TABLE 5

Partition of average nitrogen intake per rat during 70 days.

FAT CONTENT OF DIETS	INTAKE OF N	OUTPUT OF N IN		N RETAINED [1]	N RETAINED [2]
		Urine	Feces		
%	*gm*	*gm*	*gm*	*gm*	*gm*
2	6.48	2.89	0.85	2.74	2.55
30	6.31	3.04	0.71	2.56	2.56

[1] Nitrogen in food minus nitrogen in the excreta.

[2] Nitrogen in bodies of rats fed for 70 days minus nitrogen in the bodies of the control group.

Therefore there was an average of nearly 8 gm of fat synthesized by the rats on this diet. The ether extraction procedure was employed to separate the body residue into 2 portions for the purpose of quantitative grinding and sampling. No effort was made to determine precisely the true fat gains since the weights of body fat were not of primary significance.

A summary of the nitrogen utilization data and the partition of the nitrogen intake are presented in table 5. The reduction in protein content of these diets in comparison with the earlier work resulted in a considerably decreased growth rate and nitrogen retention. However, the difference in fat

content of the diets was found to exert no significant influence on the nitrogen retention. This finding is in accord with that of the former work which indicated that the influence of large amounts of fat in the diet on the nitrogen utilization was not very definite.

The recovery of the feed nitrogen in the feces, urine, and body gain was, on the average, 97 and 100% of the intake for the 2% and 30% fat diets, respectively. The comparison of nitrogen retained as calculated from intake and excretion, and the nitrogen of the body gain were in close agreement.

From the data in table 6 it is evident that the difference in fat content of the diets resulted in a significant difference in heat production. The high fat animals produced 2.4% less

TABLE 6

Partition of average daily intake of food energy per rat during 70 days.

FAT CONTENT OF DIETS	GROSS ENERGY INTAKE	ENERGY INTAKE				ENERGY OUTPUT			ENERGY RETAINED
		Protein	Carbohydrate	Fat	Metabolizable	In feces	In urine	As heat	
%	Cal.	Cal.	Cal.	Cal	Cal	Cal.	Cal.	Cal.	Cal.
2	2230	233	1886	111	2132	62	37	1894	238
30	2230	226	837	1167	2112	88	31	1849	263

heat than the pair-mates on the low fat diet. The odds that this difference is not due to chance are 280:1.

Slightly more energy was excreted in the feces and less in the urine of the animals on the high fat diet as compared to the low fat group but the resulting metabolizable energy values were essentially the same.

In general, the effect of reducing the protein content of the diets from about 22%, as fed to growing rats in the previous work, to 7% in the present study was mainly in degree rather than in nature. Despite the lower protein content of the diets the high fat ration exerted a favorable effect upon the energy utilization, demonstrated by an increase in body gain and a decrease in heat production. It seems probable from this evidence that the level of dietary protein is not a factor in the

superior energy utilization of high fat diets exhibited by growing albino rats under conditions of normal cage activity.

SUMMARY

An investigation of the possible influence of low protein intake on the superior energy utilization of high fat diets was conducted using 12 litter-mate pairs of weanling male albino rats subjected to a 70-day metabolism and body analysis procedure.

A comparison was made between 2 diets containing 2% and 30% of fat, respectively, so prepared and fed as to supply to each rat of a litter-pair and therefore to each group of 12 the same quantities of energy, protein and vitamins.

Determinations were made of gains in live weight, nitrogen, fat and energy, and of the heat production for 70 days as the energy of the food minus the energy of the excreta and of the body gain.

Decreasing the protein intake from the former 22% level to 7% of the diet did not alter the previously reported superior energy utilization of high fat diets by the growing rat. In the present experiment an increased weight gain, increased body gains of fat and energy, and decreased heat production were associated with the high fat ration.

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EFFECT OF THE PTEROYLGLUTAMIC ACID INTAKE ON THE PERFORMANCE OF TURKEYS AND CHICKENS[1]

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Previous work has shown that the young turkey requires a dietary source of pteroylglutamic acid (folic acid) and that a deficiency of this factor in the diet results in retarded growth, moderate anemia, cervical paralysis and a high mortality (Richardson et al., '45; Jukes et al., '47; Russell and Taylor, '47). Available data indicate that 0.8–2.0 mg of pteroylglutamic acid/kg of ration is required for the young poult (Jukes et al., '47; Russell and Taylor, '47).

In a recent report by Taylor ('47) data have been presented on the influence of the amount of pteroylglutamic acid (PGA) in the diet of the White Leghorn on egg production and hatchability. It was concluded that 0.12 mg/kg of PGA or less was required for high egg production but that more than this amount was needed for sustained hatchability. No work has been reported, however, on the effect of the amount of this vitamin in the diet on the performance of adult turkeys for egg production or hatchability.

In the present study the influence of the amount of PGA in the diet on the performance of adult turkeys and chickens and of young poults has been determined. Data have also been

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obtained during the past 2 seasons on the amount of PGA found in the egg and in the blood of both chickens and turkeys as influenced by the dietary intake, the survival and performance of young poults obtained from groups of hens fed diets high and low in PGA, and egg production and hatchability. The results obtained in these experiments are reported in this paper.

EXPERIMENTAL

Care of experimental birds. Broad-breasted, bronze turkey hens that were 6 months of age were placed in breeding pens at an average initial weight of approximately 7.7 kg. Eight birds were used in each of 2 groups; 1 received a diet designed to be low in PGA and the other received the same diet to which 2.0 mg PGA/kg were added. Insofar as possible the birds were distributed uniformly between the 2 groups. The houses were lighted at all times and good egg production was achieved in a period of 2 weeks. The hens were then trap-nested and records obtained on egg production. At uniform intervals throughout the experiment studies were also conducted on the hatchability of the eggs and on the amount of PGA in the egg. A similar allocation of experimental birds was used in each of the 2 seasons that this study was carried out.

First-year White Leghorn pullets (hereafter referred to as hens for simplicity) were used in parallel investigations. Fifteen hens were used in each group, 1 group receiving the basal ration and the other the PGA supplemented ration. In all cases matings were carried out with males maintained on a standard ration and insofar as possible the males were rotated among several groups of hens at regular intervals.

A series of studies was also carried out with young poults in which the effect was determined of the amount of PGA ingested by the hens on the survival and growth of the young poults fed diets deficient or adequate in the vitamin. In this work day-old poults both from the deficient and supplemented hens were used. Poults hatched from eggs laid by each hen were distributed uniformly among the experimental groups.

Composition of rations. The composition of the rations fed the adult turkeys is given in table 1. The basal ration was designed to be low in PGA and adequate insofar as possible in other nutrients. The control group was fed, in addition, 2.0 mg of PGA/kg of ration. The B vitamin supplements were prepared in the following manner. An aqueous suspension of the vitamins was added to finely ground corn, dried, reground to a fine powder and thoroughly mixed with additional ground corn so that 1% of the mixture supplied the correct amounts of the vitamins in the ration. The adult chickens were fed a similar ration, differing only in that 2% less casein was used with a corresponding increase in the amount of cerelose.

In preliminary tests conducted during 1946 a ration similar to that just described, except higher in protein, was used in studies with the young poult. In 1947, however, a purified ration of known PGA content was used. The effect of the diet of the hen on the performance of the young poult could, therefore, be more accurately evaluated. The composition of the purified ration used in this work with the young poults is also given in table 1. The amount of PGA added to the ration was varied, being 0.2, 0.8 or 2.0 mg/kg. These levels were used in order to determine the effect of feeding amounts either below, approximating or exceeding the reported requirement.

Methods of analysis. The pteroylglutamic acid content of the eggs was determined microbiologically with the use of *S. faecalis* R as the test organism (Teply and Elvehjem, '45). The vitamin was liberated from the samples by takadiastase treatment in a manner similar to that used for animal tissues (Schweigert et al., '46). The entire content of each egg was weighed and homogenized in a Waring blendor with an equal weight of water. An aliquot of the homogenate (equivalent to 5 gm of egg) was then diluted to approximately 75 ml, adjusted to pH 4.5 with HCl and sodium acetate, and incubated with 100 mg of takadiastase for 16–18 hours at 37°C. After autoclaving for 5 minutes to inactivate the enzymes, the samples were diluted to an appropriate volume, neutralized, filtered and aliquots taken for assay.

TABLE 1

Composition of rations.

RATIONS FOR ADULT TURKEYS				RATIONS FOR TURKEY POULTS			
	%		*mg/kg*		%		*mg/kg*[1]
Ground yellow corn	65.54	Thiamine	6.0	Casein	25	Thiamine	6.0
Casein	10	Riboflavin	6.6	Gelatin	10	Riboflavin	6.6
Raw bone meal	8	Pyridoxine	6.6	Corn oil	4.25	Pyridoxine	6.6
Cerelose	11.4	Ca pantothenate	22	Salts[2]	5.0	Ca pantothenate	22
Cellulose	2	Nicotinic acid	50	Fortified A and D oil	0.75	Nicotinic acid	50
Oyster shell	2			Cystine	0.2	Inositol	1000
NaCl	0.6	Pteroylglutamic acid	0 or 2.0	Starch	54.8	Choline	2000
$MnSo_4 \cdot 4H_2O$	0.013					p-aminobenzoic acid	2.0
Choline	0.17					Biotin	0.2
Fortified A and D oil	0.25					2-methyl 1,4 naphthoquinone	20
Delsterol	0.026					Mixed tocopherols	100
						Pteroylglutamic acid	0.2, 0.8 or 2.0

[1] Wilkening and Schweigert ('47).
[2] Hegsted et al. ('41).

A series of determinations conducted during the 1946 season indicated that the amount of PGA added to the diet did affect the amount in the eggs. Therefore, during the present season, 8–15 eggs were taken from each group of chickens and turkeys at regular monthly intervals for PGA analysis.

At the conclusion of the feeding tests conducted with adult chickens and turkeys, samples of blood were drawn for determinations of free and combined PGA (Schweigert and Pearson, '47). The feed was withheld from the birds for 3 hours prior to taking of the blood samples. Six to 8 ml of blood were drawn from the turkeys from the wing vein and from the chickens by heart puncture into oxalated tubes. Determinations were made of the "apparent" free folic acid in whole blood and plasma, and in some cases of the amount present after enzymatic digestion on both plasma and whole blood.

RESULTS AND DISCUSSION

No effect attributable to feeding the diet low in PGA to adult turkeys or chickens was observed on general appearance, mortality, hemoglobin level, or weight change. Furthermore, no appreciable difference was noted in egg production and hatchability for the 2 species. The egg production for the group of 8 turkeys fed the diet low in PGA totalled 1108 and the egg production for the supplemented group totalled 1067 for the 2 seasons. Egg production for each group of 15 chickens fed the 2 corresponding diets was 1928 and 1945, respectively. Thus, it appears that sufficient PGA was present in the basal diet as judged by these criteria. By microbiological analysis, the basal ration was shown to contain 0.42 mg of PGA/kg of ration.[2]

It is significant to note that Taylor ('47) observed that 0.12 mg of PGA/kg was sufficient for satisfactory egg production and somewhat more was needed for hatchability. It would seem, therefore, that the requirement for good hatchability was between 0.12 and 0.42 mg of PGA/kg.

[2] We are indebted to Dr. T. H. Jukes and Dr. E. L. R. Stokstad for this analysis. A chick pancreas enzyme preparation was used to liberate the PGA.

The level of PGA in the diet does, however, markedly influence the amount of the vitamin present in the egg. Preliminary results were obtained on eggs from turkeys maintained for 5–6 months on the experimental regimen described and those from chickens maintained for periods of 1–2 months during the 1946 season. Twenty turkey eggs from the low PGA group averaged 0.113 µg of PGA/gm; 26 eggs from the PGA supplemented group averaged 0.195 µg of PGA/gm, while 13 eggs from hens fed the standard flock ration averaged 0.171 µg of PGA/gm. Separate studies showed that most of the vitamin was present in the egg yolk: 0.47 and 0.31 µg of PGA/gm of yolk for the supplemented and deficient groups, respectively; and 0.013 and 0.007 µg of PGA/gm of egg white, respectively.

Eggs from chickens fed the basal and supplemented diets for 1 month were identical in their PGA content: 0.067 and 0.064 µg/gm of whole egg; however, eggs obtained after 2 months of feeding averaged 0.12 and 0.20 µg of PGA/gm, respectively.

Experiments conducted during 1947 were designed to obtain eggs from each group at regular intervals from the beginning to 7½ months (table 2). Unfortunately, egg production was not at a high enough rate to merit analyses prior to 1½ months on experiment. It will be noted that a difference was obtained in the content of PGA in 1½ months of feeding. The difference in the amount found in the eggs did not appear to become greater during the test period. Thus the mean difference was as great at 2½ or 3½ months of feeding as it was at 6½ or 7½ months. Insofar as possible, determinations were made on eggs from each hen each month; therefore each figure is the mean obtained for determinations on 8–15 eggs. The number in each group was regarded as insufficient for a separate statistical analysis; therefore the results for the entire period were analyzed statistically. It was shown that the effect of the level of PGA ingested on the amount found in the eggs of both chickens and turkeys was highly significant.

It is recognized that the techniques used for the liberation of PGA from the eggs may not have resulted in complete liberation of the vitamin. Some studies were conducted with the use of chick pancreas enzyme [3] or a hog kidney preparation (Bird et al., '46). Higher values were obtained with the use of these preparations in some cases; however the relative difference in the values for the eggs from the supplemented groups as compared to those from the low PGA group was

TABLE 2

Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of this vitamin found in the egg (1947 season).

LENGTH OF TIME ON EXPERIMENT	TURKEYS		CHICKENS	
	Basal ration	PGA supplemented ration	Basal ration	PGA supplemented ration
Months	*µg PGA/gm of whole egg*	*µg PGA/gm of whole egg*	*µg PGA/gm of whole egg*	*µg PGA/gm of whole egg*
1½	0.13	0.19	0.08	0.11
2½	0.09	0.15	0.09	0.16
3½	0.09	0.12	0.08	0.12
4½	0.16	0.30	0.15	0.17
5½	0.16	0.30	0.08	0.12
6½	0.06	0.18	0.11	0.13
7½			0.11	0.14
Total no. of eggs	44	43	59	59
Mean and S.E.	0.117 ± .007	0.209 ± .013	0.098 ± .004	0.133 ± .005
F Value	42.9 [1]		39.7 [2]	

[1] F Value required for significance at the 1% level is 6.93.

[2] F Value required for significance at the 1% level is 6.86.

consistent with the results obtained with takadiastase treatment. Therefore, in order to obtain comparable data during the experiment, the takadiastase technique was used throughout, and since equal numbers of eggs from each group of birds were included in each series of determinations, the relative values obtained are assumed to be valid. Variations in the amount found for any 1 month may be due in part to seasonal effects attributable to the level of egg production, feed con-

[3] Dr. L. R. Richardson generously supplied this preparation.

sumption, etc., but the figures may also represent variations in the amount of the vitamin liberated in the specific series of analyses carried out.

Blood studies. Analyses obtained on the "apparent" free PGA content of whole blood and plasma also demonstrated the influence of the dietary treatment. Results were obtained at the end of the experiments for both seasons on adult chickens and turkeys. In some cases, blood was taken from the same

TABLE 3

Effect of the amount of pteroylglutamic acid ingested by turkeys and chickens on the amount of "apparent" free pteroylglutamic acid found in the blood.

LENGTH OF TIME ON EXPERIMENT	SAMPLE USED	MILLIMICROGRAMS OF PGA/ML.[1]		F VALUE	
		Basal ration	PGA supplemented ration	Observed	Required (1% level)
		Turkeys			
1946					
6 months	whole blood	9.0(12)	16.8(12)	17.8	7.88
	plasma	1.7(8)	6.0(8)	127.5	8.68
1947					
7 months	whole blood	8.4(16)	15.9(16)	19.2	7.53
	plasma	2.5(16)	7.4(16)	25.1	7.53
		Chickens			
1946					
2½ months	whole blood	10.2(14)	11.0(14)	not significant	
	plasma	3.2(13)	4.8(13)	13.0	7.77
1947					
7½ months	whole blood	9.7(8)	8.3(10)	not significant	
	plasma	3.8(8)	4.5(9)	not significant	

[1] The number of analyses is given in parentheses.

birds 2 weeks prior to the end of the experiment. These results are presented in table 3. The amount of "apparent" free PGA in the whole blood of the turkeys, for example, is significantly lower for the group fed the basal ration (8.6 mμg/ml) than for the group fed the supplemented ration (16.2 mμg/ml). In some cases the amount of free PGA in the cells and plasma for the 2 groups was determined. An average of 27.8 and 41.4 mμg of PGA per milliliter of cells from the defici-

ent and supplemented groups, respectively was found; 1.0 and 6.0 mμg of PGA per milliliter of plasma, respectively.

No difference was noted in the values for whole blood of the chickens between the 2 groups. The values for plasma reflected the influence of the dietary treatment in tests conducted during 1946, but no significant difference was noted in those conducted in 1947. More data are needed to explain this apparent discrepancy: however uncontrolled variables such as egg production, feed consumption and time of the year may have exerted an effect. Nevertheless, the data do demonstrate that the level of the microbiologically available vitamin in the blood of the chicken is relatively insensitive to the amounts of PGA fed, as compared to that available in the turkey. The values for the supplemented groups obtained in the present study are in excellent agreement with those obtained in previous work on the "apparent" free folic acid content of blood from various species (Schweigert and Pearson, '47). The higher values observed for the amount of the free vitamin in the blood of the avian species as compared to those obtained for others may be correlated with the higher requirement of the avian species for this vitamin.

Analyses obtained after enzymatic digestion were similar for whole blood and plasma for the 2 experimental groups. The following values were obtained for the amount of PGA in whole blood after enzymatic treatment for the basal and PGA supplemented turkeys: 56.1 mμg/ml (average of 16 determinations) and 64.7 mμg/ml (average of 16 determinations), respectively. For plasma, 57.9 and 57.8 mμg/ml were observed, respectively, for the 2 groups (14 analyses in each case). The amounts observed in chicken blood were as follows: 53.8 and 55.5 mμg/ml of whole blood for the basal and supplemented groups (average of 20 determinations in each case) and 38.9 and 36.1 mμg/ml of plasma, respectively (average of 14 analyses in each case). These rather surprising observations may be of great importance. It may be possible that animals that are very deficient in this vitamin may have sufficient amounts of the conjugated form (presumably that incre-

ment measured after enzymatic digestion) present in the blood and other tissues and are unable to break it down to the biologically active or free form. Work along this line will be conducted to determine the significance of this preliminary observation. From the data obtained on whole blood and plasma, it is apparent that most of the free vitamin occurs in the cells, while the amount of PGA observed after enzymatic digestion appears to be the same for both plasma and cells.

The greater differences noted in the PGA content of the eggs and blood for the 2 groups of turkeys as compared to the 2 groups of chickens suggest that the requirement of this vitamin is greater for the turkey than for the chicken.

Studies with the young poults. Preliminary experiments conducted in 1946 indicated that poults from hens receiving the low PGA diet grew at a slower rate than the poults from the adequately supplemented hens when the poults were fed rations similar to those fed the hens. In another experiment, a low PGA diet, high PGA diet and the standard flock ration were fed to 10 poults in each group. These poults were from hens fed an adequate ration. At about 4 weeks of age all birds in the low PGA group became infected with fowl pox, while those in the other 2 groups (located above and below the basal group in the brooders) did not become affected. Since this disease is considered very contagious, it seemed rather surprising that only the low PGA group was affected unless the type of ration fed influences the susceptibility. Much more work with carefully controlled conditions will be needed to verify this observation.

Poults hatched from eggs obtained after the breeding turkeys had been on experiment for 4, 5 and 6 months during 1947 were used to determine the effect of the dietary intake of the hens on the performance of the young poult (table 4). Purified rations were used in which the PGA content was varied from 0.2–2.0 mg/kg for the various groups (table 1). In the first experiment poults from hens that received either the basal ration or supplemented ration were used. All 9 poults from hens receiving the former ration died within 2 weeks when

fed 0.2 mg of PGA/kg of ration. However, all poults fed 2.0 mg/kg, regardless of the diet fed the hens, lived and grew at a normal rate. Three of 5 poults from hens fed the supplemented diet died when 0.2 mg of PGA/kg was fed and the remaining 2 poults grew normally. These observations were extended in 2 subsequent experiments in which groups were fed 0.8 mg of PGA/kg of ration in addition to groups fed 0.2 or 2.0 mg/kg. These results are summarized in table 4. It

TABLE 4

Effect of the amount of pteroylglutamic acid in the diet of the turkey hen and poult on the performance of the young poult.

EXP. NO.	PGA ADDED TO DIET		NO. OF POULTS	WEIGHT OF POULTS				NO. OF DEATHS[1]	NO. WITH PEROSIS
	hens	poults		1 day	1 wk.	2 wks	3 wks		
	mg/kg	*mg/kg*		*gm*	*gm*	*gm*	*gm*		
2	none	0.2	18	59	77	121	194	8	4
	2.0	0.2	7	57	78	145	234	none	2
	none	0.8	15	56	72	125	222	3	6
	2.0	0.8	6	58	89	173	285	none	2
	none	2.0	16	59	74	125	213	1	7
	2.0	2.0	7	61	81	146	250	1	1
3	none	0.2	6	56	85	125	199	2	1
	2.0	0.2	5	53	89	159	292	none	3
	none	0.8	6	54	89	225	332	none	2
	2.0	0.8	5	52	89	213	311	none	none
	none	2.0	5	59	84	149	298	none	none
	2.0	2.0	4	54	84	165	327	none	none

[1] Only those poults that died after 4 or more days on experiment are included in this tabulation.

will be noted that the mortality and also the rate of growth of the young poults were influenced by the diet of the hens from which the poults were obtained. From these experiments it can be seen that a total of 10 out of 24 poults died prior to 3 weeks of age when both the hens and poults received the diets low in PGA. However, either when the hens received the PGA supplemented diet or the poults received 2.0 mg of PGA/kg of ration, few losses occurred (2 poults out of 55). The mortality observed when 0.8 mg of PGA/kg of ration was fed to the poults was intermediate when the hens had been

fed the basal ration (3 out of 19). As indicated above, an excellent performance was noted when 0.8 mg of PGA/kg was fed and the hens had been fed the supplemented ration. The rates of growth observed also reflected the dietary treatment of the hens as well as that of the poults. The requirement of the poults appeared to be between 0.8 and 2.0 mg/kg of PGA judged on the basis of rates of growth and mortality when the hens had been fed diets low in this vitamin. It appears that 0.8 mg of PGA/kg is adequate for the poults when the hens had been fed a diet to which 2.0 mg of PGA had been added. Thus, these results may form a basis for explaining the differences in the requirements for PGA reported by Jukes and associates ('47) and Russell and Taylor[1] ('47). The present work suggests that differences in the PGA nutrition of the hens may be a contributing factor in determining the amount required by the young poult.

Three of the poults from the hens fed the low PGA diet developed typical cervical paralysis (Richardson et al., '45; Jukes et al., '47) at 17–19 days of age when receiving 0.2 mg of PGA/kg. No other groups demonstrated this syndrome. The incidence of perosis was also higher for the poults from hens receiving the diet low in PGA, particularly in the second experiment. Thus, it has been demonstrated that feeding diets low in PGA to the hen adversely affects the ability of the young poult to survive and grow, and also increases the amount of the vitamin needed in the diet of the poult for normal development. These observations suggest that some of the mortality observed in young poults is due to subminimal levels of various nutrients, including PGA, present in the diet of the hens. Perhaps more attention should be focused on the development of improved rations for the breeding flock as well as for the growing birds.

Although no effect could be demonstrated on the egg production or hatchability due to ingestion by the hens of a diet low in PGA, the use of other criteria, namely, the levels of PGA in the eggs and blood and the performance of young poults from these hens suggests that the level of PGA fed

(0.42/kg of ration) was inadequate for the breeding turkey. The use of several criteria, therefore, in evaluating the nutritive value of the diet is highly desirable. With improvements in methods for liberating this vitamin from natural materials, more exact information on its distribution in feeds and stability will be forthcoming. Such information will facilitate more definite evaluation of the adequacy of a particular ration in terms of pteroylglutamic acid.

SUMMARY

1. The effect of feeding diets low and high in pteroylglutamic acid on the performance of adult chickens and turkeys and turkey poults was determined.

2. When a basal diet containing 0.42 mg of PGA/kg of ration was fed to turkey or chicken hens no detrimental effect on egg production, hatchability, hemoglobin level or general appearance was demonstrated as compared to when 2.0 mg of PGA were added/kg of ration.

3. The amount of PGA found in the eggs of both chickens and turkeys was markedly lower when the basal diet was fed as compared to the amount found when the diet supplemented with PGA was fed.

4. The "apparent" free folic acid content of the blood was also lower for the turkeys fed the low PGA diet while the level in the blood of the chickens was relatively insensitive to differences in the dietary treatment. The amount of PGA observed after enzymatic digestion of the blood samples, however, was shown to be approximately the same for both groups of chickens and turkeys, regardless of dietary treatment.

5. A higher mortality and slower rate of growth were noted when both young poults and the hens were fed diets low in pteroylglutamic acid than when a supplemented diet was fed either to the hens, to the poults, or to both.

6. For poults from hens adequately supplemented with PGA, 0.8 mg of the vitamin/kg appeared to be adequate; however, for poults from hens fed the diet not supplemented

with PGA, the requirement is apparently greater than 0.8/kg of ration.

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THE RELATION OF FED AND INJECTED TOCOPHEROLS TO DEVELOPMENT OF RANCIDITY IN THE STORED MEAT AND UTILIZATION OF CAROTENE BY THE RABBIT[1]

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Work has been done to show that alpha-tocopherol, which prolongs the induction period of the fat, is stored in the tissues of the rat and turkey (Criddle and Morgan, '47; Hanson et al., '44; Lundberg et al., '44; and Mason, '42). Watts, Cunha and Major ('46) found that the amount of tocopherol stored in hog fat was too small a quantity to be of practical value, especially when the animals were fed a natural ration.

In the work reported here, a study was made of the relation of the tocopherols to the prevention of development of rancidity in the stored meat and fat of the rabbit. In addition, the effect of tocopherols upon the utilization of carotene was investigated.

A number of authors (Davies and Moore, '41; Hickman et al., '42, '44a, '44b; Moore, '40; and Jensen, '46) have reported a sparing action of tocopherol upon vitamin A. Guggenheim ('44), Hove ('43, '44), Jensen et al. ('43), Quackenbush et al.

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('41, '42), and Sherman ('42) have shown also that the tocopherols are effective in enhancing the utilization of carotene. Harris et al. ('44) found that 0.5 mg of natural mixed tocopherols was the optimum daily dose to demonstrate the sparing action of carotene on rats.

METHODS

Selection and care of animals

American white rabbits weighing 500–700 gm were used for this experiment. They were weaned when 4 to 6 weeks old and fed commercial rabbit pellets until all were eating, as evidenced by maintenance of weight. They were then separated according to sex and put into 3 comparable groups, and placed on the experimental diet. Supplements to the diet were withheld until the vitamin A stores were depleted, as evidenced by leveling off of the growth curve.

Diet and supplements

The composition of the purified diet is shown in table 1. Contrary to the findings of Mackenzie, Mackenzie and McCollum ('41), preliminary work with rabbits in our laboratory showed that fat was essential in the diet, both for utilization of carotene and general health and growth. For this reason lard was included in the diet.

Sufficient diet for 1 week was prepared at one time by mixing thoroughly all of the ingredients of the diet and then moistening the mixture sufficiently to form a pellet. The slightly moist pellets were spread on screens at room temperature overnight to dry and stored in closed tin cans for the week. The peroxide values of the diet at the beginning and end of the week were followed until it was apparent that there was no appreciable increase during this period. At first the yeast was ether-extracted, but this practice was soon discontinued because of the extremely low tocopherol content (Veron, '46). The diet was fed *ad libitum* in metal containers.

Supplements containing carotene, viosterol, and mixed tocopherols were prepared for each group weekly. Crystalline carotene (90% beta and 10% alpha) was dissolved in chloroform in 1-gram lots; the solution was taken up in ethyl laurate; and the chloroform was then evaporated on a water bath. The volume of the carotene solution was adjusted with ethyl laurate so that 1 ml of the solution contained 20 mg carotene. It was then stored in brown bottles in the refrigerator. Aliquots of this solution were used in mixing the weekly supplement. The vitamin D used was viosterol in oil with

TABLE 1

Composition of rabbit diet.

BASAL DIET		SUPPLEMENTS	Tocopherol low	Tocopherol high
	%		*mg/day*	*mg/day*
Casein	15	Tocopherol	1.0	11.1
Brewers' Yeast	15	Viosterol	1.0	1.0
Lard	10	Carotene	3.0	3.0
Wheat Bran	15			
Salt Mix [1]	6			
Cornstarch	39			

[1] Salt mix no. 51 (Mackenzie, Mackenzie and McCollum, '39).

standardized potency of 10,000 U.S.P. units per gm. A tocopherol concentrate containing 34% mixed tocopherols was used.

For the groups fed high levels both the viosterol and tocopherol were weighed directly into brown bottles, the carotene solution added, and the volume adjusted with ethyl laurate so that 0.3 ml was fed at each supplementation. The mixed supplement was then stored in the refrigerator. This supplement was fed by pipette directly into the animal's mouth 3 times weekly.

For the groups of animals receiving injected tocopherol, the viosterol and carotene were prepared and fed according to the method followed for the previous groups. The tocopherol, however, for this group was weighed out and the volume adjusted with ethyl laurate so that 0.5 ml contained the weekly

supplement which was injected with hypodermic syringe into the heaviest portion of the right thigh once weekly. All injections were kept within a 1-inch circle on the thigh.

The daily tocopherol requirement of the rabbit has been found to be between 0.2 mg and 1.0 mg per kilogram body weight (Eppstein and Morgulis, '40, '42; Friedman and Mattill, '41; Hove and Harris, '47; Mackenzie, Levine and McCollum, '40; Mackenzie and McCollum, '39, '40). The amount of 1.0 mg mixed tocopherol was chosen for the low level group to avoid any incidence of dystrophy.

Treatment of the meat

At the end of the experimental period, the rabbits were dressed, the livers and free fat removed and frozen, and the meat cut from the bones and frozen. The right thigh of the animals which had had the tocopherol injected was removed and stored separately. After freezing, the meat of each animal was ground and the sausage mixed thoroughly. It was found much easier to get a fine grind, free from stringy tissue, if it was ground while still frozen. The sausage was weighed out into 50 gm lots, which were made into balls of similar size and shape and placed in freezer cartons with strips of wax paper between them to facilitate removal in individual balls for analysis.. The cartons were then put into frozen storage at —18°C.

Rancidity tests on the frozen sausage

The samples were analyzed at intervals until the sausage from each animal was found rancid. The fat was extracted from the sausage by blending 50 gm sausage in a Waring blendor for 1 minute with 100 mg chloroform and 70 gm of anhydrous sodium sulfate. The extract was filtered through Whatman no. 2 filter paper, and a 5 ml sample of the clear filtrate dried to determine fat content. The amount of the filtrate necessary to make 0.5 gm fat was used for the test. A peroxide value above 10 was designated rancid. The

method used for determination of peroxide values throughout the experiment was reported by Watts and Major ('46).

Vitamin A and carotene analyses

Vitamin A analyses were made on all livers using the Carr-Price reaction as reported by Davies ('33). This was changed to the Gallup and Hoefer method ('46) when good agreement was found between the two. Carotene was determined by the Wall and Kelley method ('43).

Determination of pH

Five grams of sausage were mixed thoroughly with 50 ml distilled water. After 30 minutes the pH was read on a Beckman meter.

EXPERIMENTAL RESULTS AND DISCUSSION

This work was carried out in 2 separate experiments. Experiment 1 included 3 groups of animals fed the purified diet. Group 1 received a low-level tocopherol supplement; group 2, a high-level tocopherol supplement; and group 3, a high-level tocopherol supplement by injection. Experiment 2 was concerned with animals fed a natural diet which was supplemented with injected tocopherol and tocopherol phosphate.

Experiment 1

Part A. This experiment was set up using the purified diet shown in table 1. In addition to the effect of fed and injected tocopherol on development of rancidity, the effect of fed tocopherol on the utilization of carotene was estimated.

The effect of tocopherol upon rancidity development in the stored meat is summarized in table 2. At the end of 12 months' storage, all but one of the samples from the group fed low level tocopherol were rancid (mean peroxide value of 14), whereas none of those from the group fed high level tocopherol were rancid (mean peroxide value of 4.4). The mean peroxide value of the group which had the tocopherol injected was

between the average of these 2 groups, or 5.6. A statistical analysis of these figures shows that the difference between the average peroxide values of the high level of tocopherol, whether fed or injected, and the low level of tocopherol is significant at the 1% level.

TABLE 2

Effect of feeding and injecting tocopherol on the rancidity development of the meat.

CODE NUMBER	TOCOPHEROL	PEROXIDE VALUES AFTER STORAGE FOR		pH OF MEAT
		12 mo.	18 mo.	
	mg/day			
32	1.0	18.0		5.50
39	1.0	8.0	13.5	5.70
45	1.0	16.0		5.70
47	1.0	14.0		5.69
		Mean 14.0		
33	11.1	7.2	14.0	6.15
34	11.1	4.0	9.0	5.55
37	11.1	3.4	5.0	5.90
40	11.1	3.0	5.0	5.50
	Injected	Mean 4.4 [1]	8.2	
35	11.1	3.0	6.5	6.00
36	11.1	7.8	9.0	6.04
38	11.1	4.0	6.5	5.85
42	11.1	5.0	5.5	5.70
43	11.1	7.0	8.5	5.70
44	11.1	7.0	9.0	5.62
		Mean 5.6 [1]	7.5	

[1] Significant at the 1% level. The method for determining significance was taken from Snedecor, G. W., *Statistical Methods*.

It has been reported that tocopherol in the diet is necessary to prevent the destruction of vitamin A and carotene in the digestive tract. More vitamin A was stored in the liver as well as more carotene excreted in the feces when the dietary tocopherol was increased (Guggenheim, '44; Harris et al., '44; Hickman et al., '42; Hove, '43). A carotene balance was run to see if the same conditions were necessary for carotene utilization for rabbits as had been reported for rats.

Before the carotene balance was started, the animals had been maintained on the same supplements for a period of 30

days or more. A 4-day period was used as a test period, and feces were collected at a stated hour daily. Feces that could not be analyzed for carotene content immediately after collection were frozen until an analysis could be made.

The results of the vitamin A storage and carotene excretion from this experiment are shown in table 3. The lower figure of carotene excreted by the group of animals fed a low level of tocopherol appeared to be due to an extremely small amount of feces on the first day of collection (4 gm as compared to a daily average of 35 gm), rather than to any difference in

TABLE 3

Effect of tocopherol on excretion of carotene and storage of vitamin A.

PART	GROUP	NO. OF ANIMALS	SUPPLEMENTS FED		CAROTENE EXCRETED (AVERAGE)	AVERAGE VITAMIN A STORED IN LIVER	
			Tocopherol	Carotene			
			mg/day	*mg/day*	*mg/animal/day*	*mg/gm*	*total mg*
A	low	4	1.0	3.0	0.943	0.020	2.116
	high	4	11.1	3.0	1.597	0.018	1.570
	injected	6	11.1	3.0	1.327	0.014	1.298
B	low	6	1.0	1.5	0.688	0.012	1.231
	high	7	11.1	1.5	0.414	0.010	1.234

carotene utilization. The carotene content of the feces from this group on the day mentioned was even lower per gram than that on other days.

There was no significant difference between the 3 groups in either carotene excretion or vitamin A storage in the liver.

Part B. Since the results of the carotene balance and of the vitamin A assay on the livers (shown in table 3, part A) did not agree with those reported by Guggenheim on rats ('44), it was thought that another set of animals should be used to investigate the effect of the amount of tocopherol fed on carotene excretion, utilization, and rancidity development in meat. Because of the high quantity of carotene excreted and vitamin A stored in the liver, it was believed that the amount fed in part A was too far in excess of the requirement

to be a good measure of the effect of tocopherol (Moore, '40). Therefore, for this trial, the carotene level was decreased from 3.0 mg daily to 1.5 mg daily. Because Fraps and Meinke ('45) and Callison and Orent-Keiles ('46) had reported that the availability of carotene was greater when fed in an oil, the supplements were mixed and fed in peanut oil [4] instead of in ethyl laurate.

The results on carotene excretion and vitamin A storage for both parts of this experiment are shown in table 3. In neither experiment did the greater amount of tocopherol fed result in significant change in amount of carotene excreted nor in the amount of vitamin A stored in the liver. The tocopherol supplement, even in the low level, may have been sufficient to exert protective action on the carotene. More work is needed before conclusions can be drawn on the effect of feeding the supplements in oil instead of in ethyl laurate. However, some fat, either in the diet or in the supplements, appeared necessary in order to get any storage of vitamin A in the liver. In preliminary work where both were omitted, no vitamin A was found in any of the livers, although 3.0 mg carotene was fed daily. This was found to be in agreement with other work (Callison and Orent-Keiles, '46; Hove and Harris, '46; Quackenbush et al., '41, '42).

An accelerated method for development of rancidity in the ground meat was devised and compared with frozen storage results. This method consisted of mixing thoroughly 4.0 ml of chloroform with 25.0 gm of the ground meat ready for freezing. The mixture was put into 250 ml beakers in a layer of uniform thickness, the beaker capped with aluminum foil to prevent vaporization of the chloroform, and stored in an incubator at 30°C. In this way these samples could be stored for periods as long as 60 hours without apparent bacterial spoilage. Peroxide values were run on samples stored in this way at 36- and 48-hour intervals.

The results of the accelerated tests on the meat are shown in table 4. At the end of the 36-hour period all but one of

[4] Obtained through the courtesy of Planters Edible Oil Company, Suffolk, Va.

the meat samples from animals fed the low tocopherol level were rancid and that one was near the point of rancidity (average 17.1). On the other hand, only one of the samples from the animals fed high tocopherol showed any development of rancidity at the end of the 36-hour period (average 2.8). Statistical analysis shows that this difference is significant at the 1% level. The early development of rancidity in the one

TABLE 4

Comparison of accelerated and frozen storage tests on sausage.

CODE NUMBER	TOCOPHEROL FED	PEROXIDE VALUES			pH OF MEAT
		Accelerated		Frozen storage	
	mg/day	*36 hr*	*48 hr*	*6 mo*	
63	1.0	13.7	19.5	11.5	5.83
66	1.0	23.2	37.0	11.5	5.80
69	1.0	26.5	41.5	21.0	5.72
70	1.0	20.0	35.0	14.5	5.60
72	1.0	8.5	22.0	8.5	5.65
74	1.0	10.5	23.5	9.5	5.73
		Mean 17.1 [1]	29.6	12.7	
61	11.1	12.5	25.0	11.0	5.73
62	11.1	0.1	1.5	1.0	5.70
64	11.1	3.5	4.3	0.1	5.72
65	11.1	0.5	0.7	3.5	5.83
68	11.1	0.0	0.5	3.5	5.70
71	11.1	3.0	4.0	4.5	5.70
73	11.1	0.0	3.7	5.0	5.68
		Mean 2.8 [1]	5.7	4.1	

[1] Significant at the 1% level.

sample cannot be explained on the basis of pH difference (Watts and Peng, '47a). A colorimetric comparison of filtrates of the meat (prepared by blending 100 gm sausage with 140 ml water) from the different animals showed no higher hemoglobin content in this sample (Watts and Peng, '47b).

Peroxide values were determined on the meat after 6 months in frozen storage. The relative values, as shown in table 4, agreed with those made earlier by accelerated tests.

Experiment 2

This experiment was set up to determine the effect of injected tocopherol and tocopherol phosphate on rancidity development in the stored meat of rabbits fed a natural diet.

The diet used was a commercial rabbit chow [5] with the following guaranteed percentage composition: crude protein, not less than 14.5%; crude fat, not less than 2.0%; crude fiber, not more than 18.0%; nitrogen free extract, not less than 44% and ash, not more than 7.5%. The ingredients were ground oats, corn meal, soybean oil meal, corn germ meal, alfalfa meal, wheat gray middlings, molasses, riboflavin supplement, 1.5% calcium carbonate, and 0.5% iodized salt.

The animals selected for this experiment were 3 to 4 months old and had nearly reached full growth. They were divided into 2 lots; each lot consisted of a control group receiving no supplements and an experimental group. Lot 1 experimental group was injected with 200 mg of mixed tocopherol in ethyl laurate at the beginning and mid-point of a 3-week period. For lot 2 experimental group, 200 mg of water-soluble tocopherol phosphate was injected at these same intervals.

At the end of the 3-week period the animals were dressed and the right thigh of the injected group separated before storage, as has been described under the method.

The results of the experiment are shown in table 5. In the frozen storage tests of the ground meat no difference was shown between the animals injected with tocopherol or tocopherol phosphate and those of the control groups receiving no supplementary tocopherol. However, when compared to the rest of the tissue, protection from rancidity development was shown, in both lots, in the thigh muscle at the site of injection of the tocopherol.

It may be that the time between the injection of the tocopherol and killing of the animals was too short to allow for storage throughout the tissues, although Eppstein and Morgulis ('42) found that a 3–10-day period was sufficient

[5] Distributed by the Ralston Purina Company.

to cure rabbits of advanced dystrophy when they administered the water soluble disodium-α-tocopherol phosphate by injection. This experiment allowed fully the 10-day period suggested by Eppstein and Morgulis. Hove and Harris ('47) also found that injected tocopherol-phosphate brought about the cure of muscular dystrophy in the rabbit in a 5–25-day period.

TABLE 5

Effect of injecting tocopherol and tocopherol-phosphate. (Mature animals on natural diet.)

CODE NUMBER	AMT. TOCOPHEROL INJECTED (MG)	STORED MEAT PEROXIDE VALUES			pH OF MEAT
	Mixed tocopherols	5 mo.	7 mo.	9 mo.	
Lot 1					
21	0.0		16.0		6.60
22	0.0		12.0		6.20
23	0.0		8.0		6.17
24	400.0		7.0		6.10
25	400.0		17.0		6.11
25 [1]				11.0	
26	400.0		5.0		6.03
Lot 2	Tocopherol phosphate				
50	0.0	2.8			6.14
51	0.0	2.8			5.90
52	0.0	15.7			5.91
53	400.0	72.7			5.96
54	400.0	20.0			5.80
54 [1]				7.5	
55	400.0	29.5			5.78
55 [1]				9.5	
56	400.0	7.2			6.02
56 [1]				4.5	

[1] Muscle at site of injection.

SUMMARY AND CONCLUSIONS

The relation of tocopherols in the diet to development of rancidity in the fat and meat and to the utilization of carotene was investigated.

1. When high levels of tocopherol were fed to or injected in rabbits on a purified diet, protection from the development of rancidity was shown.

2. On a natural ration, no protection from the development of rancidity was shown when the animals were injected with tocopherol or tocopherol phosphate 3 weeks before slaughter.

3. Accelerated tests for the development of rancidity on sausage preserved with chloroform agreed with tests made on sausage held in frozen storage.

4. No difference either in the utilization of carotene or in the liver storage of vitamin A was observed on supplementing a synthetic diet with high and low levels of tocopherol.

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THE EFFECT OF DIETARY RESTRICTION OF B-COMPLEX VITAMINS AND PROTEIN ON THE EXCRETION OF CREATININE BY HUMAN SUBJECTS[1,2]

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FOUR FIGURES

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The work of Folin ('05), Hoogenhuyze and Verploegh ('05), Klercker ('07), and Shaffer ('08) some 40 years ago led to the formulation of several concepts which, in spite of intensive investigation and much individual disagreements, still are of interest to the nutritionist: (1) that some biochemical phenomena proceed at similar, apparently constant,

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rates in all healthy physically fit individuals, quite independent of the usual variations of the normal diet and physical activities and, therefore, may be used as "yard sticks" for assessing the total active mass of certain types of tissues; and (2) that alterations of structure or functional efficiency of such tissues resulting from disease, malnutrition or other debilitating conditions may be reflected in altered rates of the basic reactions.

Although the first is opposed to modern concepts of extreme complexity and variability of biochemical phenomena, nevertheless, the early investigators demonstrated such an apparent constancy in the case of creatinine in relation to the unit weight — the creatinine coefficient — which presumably is related to the muscular mass (Shaffer, '08; Hodgson and Lewis, '26; McClugage, Booth and Evans, '31). This was all the more surprising since the conclusions were based upon determinations of the daily rate of urinary excretion which, in addition to cellular mechanisms, involves renal function. In recent years the role of protein and amino acids in the synthesis of creatine and creatinine has received particular attention. The result has been an increasing emphasis on the variation rather than on the uniformity of the creatinine excretion. The changing point of view is evident in the reviews of Hunter ('27), Peters and Van Slyke ('46) and Beard ('41).

The differences in the average daily rate of excretion were explained by Folin and Shaffer largely on the basis of the second postulate. This was perhaps best stated by Shaffer ('08) that "in a muscle in a high state of nutrition and development, certain processes which cannot be fully defined at present, but which lead to the formation of creatinine as a waste product, are proceeding at a greater speed than in a muscle organically weak or diseased" and that upon the intensity (see Hoogenhuyze and Verploegh, '05) of these processes, as measured by the creatinine coefficient, "appears to depend the muscular efficiency of the individual." Because of the experimental difficulties and the long time required in

such investigations, these implied relations have not been studied in a satisfactory manner in human subjects.

In the course of an experiment with 7 initially physically fit young men (Berryman et al., '47), an opportunity was presented for applying uniform procedures to a study of the variations of creatinine excretion and their relation to changes in weight and physical performance over a period of 50 weeks as follows: a "normal" period of 11 weeks; an experimental period of 8 months (36 weeks) during 4 of which the diet was restricted with respect to protein and B-complex vitamins, followed by 4 months in which a program of supplementation was instituted in accordance with individual needs; and a final period of 3 weeks, during which all subjects received approximately equal quantities of a rehabilitation diet. Two of the subjects served as "controls" on the effect of the restricted basal diet plus all of the supplements during the 2 periods of 4 months each. Thus, the experiment provided data on the "constancy" of the creatinine coefficient under controlled conditions and the effect of subsequent changes in the "state of nutrition."

EXPERIMENTAL

Since the experimental conditions were described fully by Berryman et al. ('47) in connection with other data, only the salient features will be given here. The 7 men, ages 22 to 27 years, were chosen from many volunteers on the basis of physical and mental qualities. All subjects received equal weighed quantities of "normal" diet I during the first 11 weeks, during which a uniform program of training and test procedures was instituted and an apparent plateau of physical performance was attained. The diet contained (Berryman et al., '47): 3170 cal.; 70 gm protein (including protein from 75 gm lean beef, 225 ml milk and 1 egg); 1.44 mg thiamine; 1.84 mg riboflavin; 15.6 mg niacin; 44 μg biotin; 64 μg L. casei factor; 4.7 mg pantothenic acid; 1.7 mg pyridoxine; and adequate quantities of ascorbic acid, vitamin A, calcium, phosphorus, and iron.

The basal weighed experimental diet II was then given to all subjects: a total of only 5 weeks (12 to 16, inclusive) to subjects 3 and 4, and 36 weeks (12 to 47, inclusive) to the other 5 subjects. This permitted a determination of the initial creatinine excretion on a relatively creatine and creatinine-free diet by the 5 experimental subjects and *subjects 3 and 4*

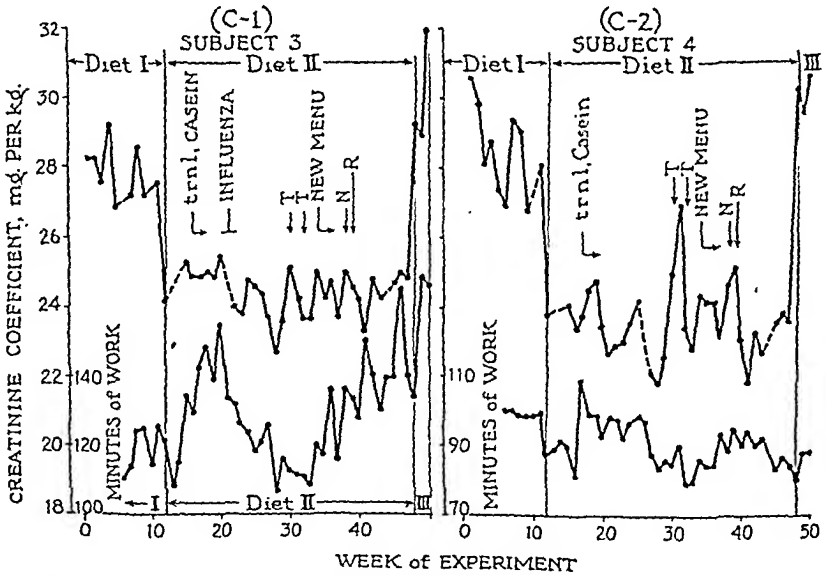

Fig. 1 Control subjects. Creatinine coefficient and work output expressed in total minutes of work to exhaustion in 2 work periods. The letters refer to vitamin supplementation — small letters to vitamins given orally in tablets 3 times per day in quantities sufficient to bring the total intake to the level of diet I; capital letters to vitamins given intravenously in large doses — as follows: t or T, thiamine; r or R, riboflavin; n or N nicotinamide; 1, "lesser known" B-complex vitamins, pyridoxine, pantothenic acid, biotin, and L. casei factor.

who subsequently served as "controls" on the effect of the basal diet plus supplements. The diet contained: 3300 cal.; 45 gm protein (including animal protein only from salt pork without visible lean meat); 0.50 mg thiamine; 0.30 mg riboflavin; 5.8 mg niacin; 19 μg biotin; 23 μg L. casei factor; 1.1 mg pyridoxine; 1.1 mg pantothenic acid; and adequate quantities of ascorbic acid, vitamins A and D, and minerals either in the diet or in supplements.

Supplements were then given to the control subjects 3 and 4: 40 gm of protein in the form of calcium caseinate and the 7 crystalline vitamins indicated in both diets above, in quantities equal to that of diet 1. These subjects later received

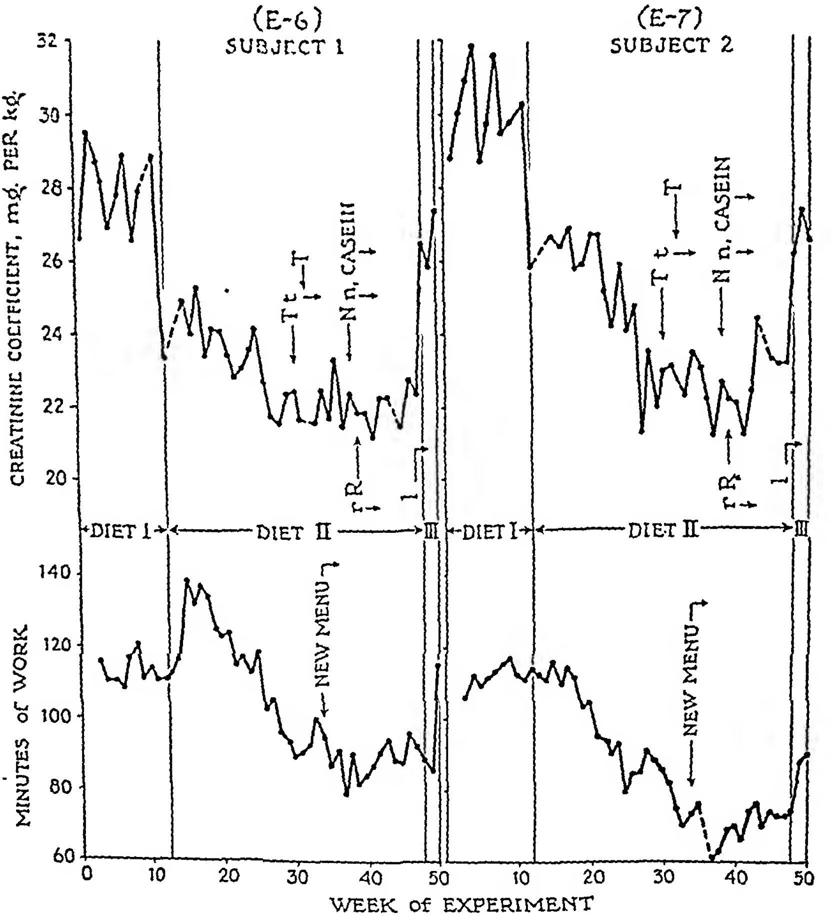

Fig. 2 Experimental subjects 1 and 2. See figure 1.

intravenous injections of vitamins at the same time as the experimental subjects (see capital letters in figures 1 to 4).

The month in which protein supplementation was instituted in the 5 experimental subjects is shown in tables 1 and 2; the

order of vitamin and other supplementation is indicated in figures 1 to 4. This had to be changed somewhat in individual cases because of the symptoms which developed in some subjects. Thus protein (casein) supplementation was instituted as follows: at the beginning of the fifth month to experimental subjects 5, 6, and 7, and at the beginning of the seventh month to experimental subjects 1 and 2. In addition, subjects 6 and 7 received further supplements of 100 gm lean

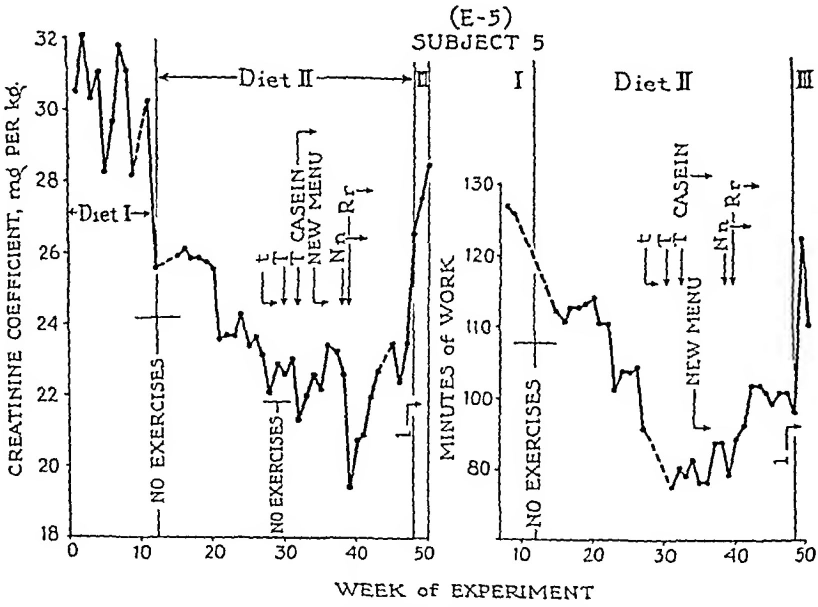

Fig. 3 Experimental subject 5. See figure 1.

beef, 225 ml milk and 1 egg at the beginning of the seventh month.

Rehabilitation diet III was given during weeks 48 to 50, inclusive. It contained 250 to 350 gm meat, 1 to 1.5 liters milk, 1 to 2 eggs, dried brewers' yeast, and, in addition, all of the crystalline B-complex vitamins. All subjects were served the same generous portions and, although they were allowed to ask for additional food, they seldom ate more. Thus, the in-

take of food, although not weighed as in the previous diets, was approximately the same for all subjects.

Weight. This was determined each morning after urine had been voided at 7 A.M. and before any water had been taken.

Work output. This was determined with a calibrated electro-dynamic brake bicycle (Kelso and Hellebrandt, '34). Work was performed twice to the point of exhaustion of the leg

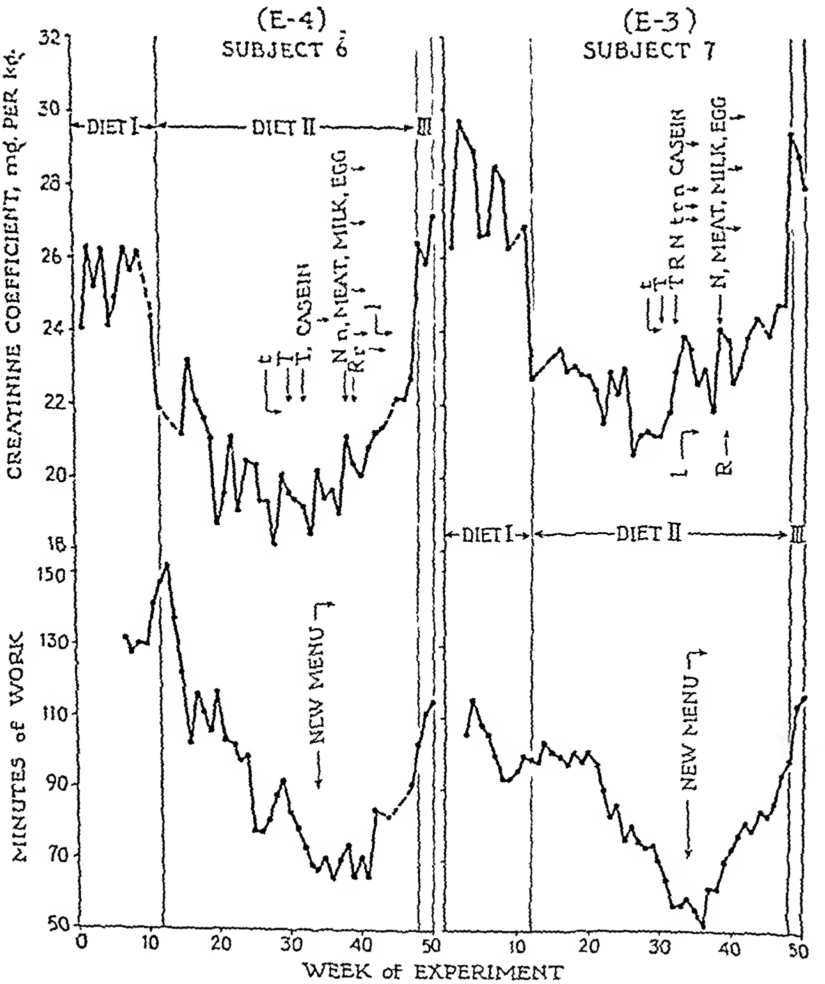

Fig. 4 Experimental subjects 6 and 7. See figure 1.

muscles, with a rest period of exactly 10 minutes between the tests (Foltz, Ivy and Barborka, '42). Previous experience had shown that the rate of pedaling is very important in obtaining comparative results from many subjects. Accordingly, throughout the year all subjects pedaled at a rate of 92 ± 4

TABLE 1

Effect of normal and rehabilitation diets. Monthly averages.

SUBJECT NO	NORMAL DIET I			REHABIL DIET III	DIFFERENCES BETWEEN RESULTS OF I AND III	
	July Weeks 1–2	Aug. Weeks 3–7	Sept. Weeks 8–11	June Weeks 48–50	Sept. and June	
	Creatinine, gm per day					
					mg	%
3	2.27	2.15	2.13	2.41	+ 280	+ 13.1
4	2.04	1.87	1.84	2.02	+ 180	+ 9.8
1	1.76	1.77	1.73	1.69	— 40	— 2.3
2	2.16	2.21	2.14	1.88	— 260	— 12.1
5	2.21	2.08	2.04	1.89	— 150	— 7.4
6	2.16	2.13	2.12	2.18	+ 60	+ 2.8
7	1.96	1.89	1.80	1.93	+ 130	+ 7.2
	Creatinine coefficient, mg per kg per day					
3	28.3	27.6	27.9	30.0		
4	30.3	28.1	28.0	30.2		
1	28.1	28.1	27.8	26.6		
2	29.4	30.5	29.8	26.7		
5	31.3	30.2	29.8	27.5		
6	25.2	25.4	25.5	26.5		
7	28.2	28.0	27.1	28.6		
	Weight, kg					
					kg	%
3	80.1	77.9	76.8	80.2	+ 3.4	+ 4.4
4	67.4	66.5	65.8	66.9	+ 1.1	+ 1.7
1	62.8	62.5	62.3	63.6	+ 1.3	+ 2.1
2	73.5	72.3	71.7	70.3	— 1.4	— 2.0
5	70.5	68.8	68.3	68.8	+ 0.5	+ 0.7
6	85.7	84.0	83.2	82.2	— 1.0	— 1.2
7	69.8	67.6	66.6	67.3	+ 0.7	+ 1.1

TABLE 2

Effect of restricted diet and supplements. Monthly averages.

The initiation of protein supplementation is indicated by *c* (45 gm casein) and *m* (100 gm lean beef, 225 ml milk, 1 egg). The numbers in parentheses in the next to last column indicate the month in which the minimum was attained; for example, (1) and (2) represent January and February, respectively.

SUBJECT NO	OCT Weeks 12–16	NOV. Weeks 17–20	DEC Weeks 21–24	JAN Weeks 25–29	FEB Weeks 30–33	MARCH Weeks 34–37	APRIL Weeks 38–41	MAY Weeks 42–46	CHANGE Jan.	CHANGE Minimum	CHANGE May
	Creatinine, gm per day								% of initial (Oct.=100%)		
3	1.90	1.91*c*	1.83	1.81	1.82	1.85	1.86	1.91	− 4.7	− 4.1(1)	+ 0.5
4	1.56	1.57*c*	1.49	1.46	1.58	1.54	1.52	1.51	− 6.4	− 6.4(1)	− 3.8
1	1.51	1.51	1.44	1.40	1.35	1.38	1.37*c*	1.42	− 7.3	−10.6(2)	− 6.0
2	1.88	1.84	1.74	1.58	1.54	1.48	1.47*c*	1.59	−16.0	−21.8(4)	−15.4
5	1.78	1.75	1.59	1.51	1.42*c*	1.47	1.37	1.51	−15.2	−23.0(4)	−15.2
6	1.84	1.71	1.63	1.54	1.47*c*	1.50	1.59*m*	1.68	−16.3	−20.1(2)	− 8.7
7	1.54	1.50	1.44	1.39	1.41*c*	1.37	1.44*m*	1.56	− 9.7	−11.0(3)	+ 1.3
	Creatinine coefficient, mg per kg per day										
3	24.8	25.1*c*	24.2	23.8	24.2	24.5	24.3	24.7			
4	23.8	24.2*c*	23.1	22.7	24.6	24.0	23.7	23.4			
1	24.1	24.3	23.3	22.5	21.9	22.3	21.9*c*	22.2			
2	26.3	26.3	25.5	23.1	22.8	22.5	22.1*c*	23.3			
5	25.9	25.8	23.8	23.0	22.3*c*	22.9	20.9	22.7			
6	22.2	21.0	20.2	19.5	19.2*c*	19.7	20.6*m*	21.8			
7	23.2	22.9	22.2	21.4	22.4*c*	22.7	23.3*m*	24.2			
	Weight, kg										
3	77.0	76.3*c*	75.5	75.9	75.2	75.6	76.6	77.4	−1.4	−2.3(2)	+0.5
4	65.9	64.9*c*	64.8	64.5	64.4	64.1	64.3	64.6	−2.1	−2.7(3)	−2.0
1	62.9	62.3	62.0	62.1	61.3	61.1	62.6*c*	63.6	−1.3	−2.9(3)	+1.1
2	71.7	69.9	68.3	68.3	67.5	65.7	66.6*c*	68.3	−4.7	−8.4(3)	−4.7
5	68.8	67.8	66.8	65.7	63.6*c*	64.0	65.6	66.6	−4.5	−7.6(2)	−3.2
6	83.1	81.6	80.8	78.7	76.5*c*	76.1	77.0*m*	77.3	−5.3	−8.4(3)	−7.0
7	66.7	65.7	65.0	64.0	62.0*c*	60.5	61.6*m*	64.6	−4.0	−9.3(3)	−3.8

revolutions per minute. In order to eliminate the complication of increasing performance due to training, all subjects performed the test 3 times each week, along with other similar tests during the first 2 months. Thereafter, the activities were decreased and the work output was determined only once each week. During the first month, the field strength of the generator, or the resistance against which the work was performed, was so adjusted that each subject became exhausted within 50 to 60 seconds in each of the 2 work periods. The rate of work finally adopted was 19.1 kgm per second for subjects 1 and 5, and 23.8 kgm per second for subjects 2, 3, 4, 6, and 7.

Urine. Collections were made 4 days each week in exactly 24-hour periods, from 7 A.M. Tuesday to 7 A.M. Saturday. The 1-gallon amber glass jugs were kept in refrigerators at all times. The volume was measured each morning, and exactly one-tenth volume was placed into a bottle containing the 4-day composite sample. The latter was kept in the deep-freeze unit.

Creatinine. The photoelectric method of Peters ('42) was used. Dilutions were made to give galvanometer deflections in the range of 40 to 60 units, using the Evelyn colorimeter. A difference of 1.0 unit, from 59 to 60, represents an error of 3.2%; from 40 to 41, an error of 2.8%. In 100 consecutive duplicate analyses, the mean analytical error was well within ± 1%. The procedure was standardized at intervals and checked by means of recoveries of added creatinine, using creatinine-zinc chloride and creatinine picrate. Throughout the experiment, all determinations were made by the same analyst.

RESULTS

The most rapid changes in weight during the first period occurred in July and August while the subjects were undergoing training. However, the greatest losses were noted during the first week. This is not apparent in the average results given in table 1. Thereafter, the weight remained well within

± 1 kg during September and the succeeding 2 months of the restricted diet II (table 2).

The weight of 4 of the subjects (2, 5, 6, and 7) began to fall quite rapidly in December (the third month of the restricted diet) when early symptoms of deficiency began to appear. Diet II became monotonous, and at this time all of the subjects began to refuse some of the corn meal biscuits and the canned vegetables. The quantity of food thus left unconsumed was variable from day to day. It was small at first, but became larger during January, amounting to from 50 to as high as 800 cal. on some days, with an average of about 200 cal. The control subjects, particularly subject 3, refused as much food as the experimental subjects. They ate all of the food when a new attractive menu, which had the same ingredients as before, was given after March 1.

The work output, expressed in minutes required for exhaustion in 2 work periods, is shown in figures 1 to 4. The test is not entirely objective; it is subject to training, and periods of "staleness" and, like all physical efficiency tests, requires cooperation. However, it is improbable that any of these factors was of major importance because of the precautions taken against the occurrence of staleness and continued training response, and because of each subject's conscientious personal desire to do his best in each test. The parallel changes which were observed with other tests during the periods of restriction and supplementation were discussed in a previous paper (Berryman et al., '47).

Excretion of creatinine and the creatinine coefficient

Weekly variations. Since the variations of weight were small from week to week, the coefficient generally paralleled the total daily excretion. The results shown in figures 1 to 4 represent the average creatinine coefficients over periods of 4 days each week. Although the average tended to minimize the daily variations, considerable change was noted from week to week. The results are plotted on a large scale, which thus exaggerates the differences, and perhaps can be judged

best by the calculated coefficients from control subjects 3 and 4 during the first 2 months of diet II. Thus, the maximum deviations from the mean of all determinations during October and November were: — 3.0 and + 2.2% from subject 3; — 2.5 and + 3.3% from subject 4. In the case of subject 3, the maximum deviations remained within this range during the entire 8 months of diet II; however, they became greater in later months in the case of subject 4, the greatest differences of — 6.2 and + 11.1% of a 2-months' average occurring during February and March.

The maximum variations of the coefficients from the 2-months' mean of each subject were somewhat greater with diet I which contained creatinine. Thus, during August and September, the maximum percentage deviations, in the order of subjects given in the tables, were: — 3.1, + 5.6; — 4.5, + 4.8; — 4.8, + 3.2; — 4.8, + 3.4; — 6.0, + 3.7; — 4.9, + 3.3; — 4.9, + 6.0.

Thus, the tendency toward variations was greater in some subjects than in others, which is evident not only in our data (figs. 1 to 4) but also in those of Folin ('05), Hoogenhuyze and Verploegh ('05), and Shaffer ('08).

In many instances, the excretion increased or declined in a regular manner over periods of several weeks during the administration of diet I. These phenomena did not occur simultaneously in other subjects. Other instances of regular decline or rise were noted during the long period over which the restricted diet II was administered.

Monthly variations. Normal diet I and first month of diet II; 8 months of diet II in case of subjects 3 and 4. Because of the weekly variations in creatinine excretion, which often appeared to be rhythmic, a certain degree of variation in the monthly averages was to be expected. The total excretion generally decreased during the 3 months of diet I (table 1).

All subjects excreted considerably less creatinine after initiation of the almost creatinine-free diet II. Thus, they excreted an average of 220 to 280 mg per day less in October than in the preceding month. In the case of the control sub-

jects 3 and 4, the creatinine excretion declined gradually until it reached a minimum in January, after which, on the same dietary regimen, it increased until, in May, it attained approximately the same level as in October (table 2).

These changes were not so apparent after calculation of the creatinine coefficient, which represents the quotient of the creatinine excretion expressed in milligrams, divided by the weight of the individual expressed in kilograms. Thus, if the excretion decreases or increases at the same percentage rate as the weight, then the coefficient remains unchanged. The coefficient reflects the relative changes in these 2 variables. During August and September, in the period of diet I, the coefficient increased slightly in the case of subjects 3 and 6, probably indicating that the weight decreased more rapidly than the excretion of creatinine. On the other hand, the coefficients decreased slightly in the case of the other 5 subjects; that is, the excretion decreased more rapidly than the weight. On the average, the 7 subjects excreted 2.16% less creatinine in September than in August, and the weight declined 1.13%. The differences are greater if the results from July and September are considered. During January, the fourth month of gradual decline, in the period of diet II, control subjects 3 and 4 excreted 4.7 and 6.4% less creatinine than in October, and the weight decreased only 1.4 and 2.1%, respectively (table 2).

On the whole, the downward trend of the creatinine excretion, although seemingly parallel, was at a greater rate than the loss of weight. Furthermore, the minima were not always attained during the same month. Thus, in the case of control subjects 3 and 4, the minimum weights were attained 1 and 2 months, respectively, after the minima of creatinine excretion (next to last column, table 2). In only 1 instance (subject 7), both minima occurred during the same month; however, here also the change with respect to creatinine excretion, —11.0%, was greater than the weight, —9.3%.

The rise in creatinine excretion which occurred from February to May often was accompanied by a rise in body weight.

However, the magnitude of the changes was not always equal (table 2). Although the changes with respect to weight and creatinine may have resulted from some fundamental alterations in metabolism, the effect, even though small, was not equal. In other words, the factors concerned with formation and excretion of creatinine appear to be subject to control and variation like all other biochemical phenomena. This point must be considered in any discussion concerning the creatinine coefficient.

However, in confirmation of the observations of Folin and Shaffer, it was found that the average daily excretion over a period of 1 month was to some extent related to the average weight of the individuals. Six of the subjects were muscularly well-developed, and had no evident excess fat. Subjects 2, 3, and 7 were tall and thin; subjects 4, 1, and 5 were stocky. Subject 6 was fat. The average daily creatinine excretion in September, in the order of decreasing weight (table 1, subjects 6, 3, 2, 5, 7, 4, 1) were: 2.12, 2.13, 2.14, 2.04, 1.80, 1.84, and 1.73 gm. The creatinine coefficients in the same order of subjects were: 25.5, 27.9, 29.8, 29.8, 27.1, 28.0, 27.8. On excluding the data from subject 6 who was fat, the highest coefficient was 4.9% above, and the lowest 4.6% below, the mean of 28.4 mg per kilogram per day.

The same approximate relation was observed in October, in the same order of subjects of decreasing weight as above, when diet II was given (table 2): 1.84, 1.90, 1.88, 1.78, 1.54, 1.56, 1.51 gm average creatinine excretion per day. The creatinine coefficients were: 22.2, 24.8, 26.3, 25.9, 23.2, 23.8, 24.1. Again, by excluding the first result, that of subject 6, the maximum deviations from the mean of 24.7 were —6.1 and +6.5%.

Since the subjects ate the same weighed diets and, therefore, metabolized about the same quantity of protein, the differences cannot be ascribed to differences of "intensity" of exogenous protein metabolism, as postulated by Beard ('41). This is further evident from the variable and negligible

effect of the addition of casein to the basal diet of subjects 3 and 4 in November (table 2).

Effect of restricted diet II and supplementation. From weeks 12 to 28, none of the experimental subjects received supplements of vitamins or protein in the basal diet. Like that of the control subjects, the creatinine excretion decreased, but to a greater degree. The excretion dropped rapidly in the case of subjects 2, 5, and 6. Clinical symptoms appeared in varying degrees in all subjects, and were evidenced in part by the following signs: decline in work output, as compared with that of the control subjects, which became most marked during weeks 21 to 24 (figs. 1 to 4); altered cardiovascular response to various work tests; scrotal skin lesions in subjects 1, 5, 6, and 7; irritability, insomnia, lack of power of concentration which forced 3 of the subjects to give up courses of study in the evening school; pain in the legs and ankles; and increased pyruvic acid content of the blood. In the case of subjects 2, 5, and 6, the marked decrease in creatinine excretion began with, and paralleled, the development of the deficiency symptoms. However, this was not so evident in the case of subjects 1 and 7 when the results were compared with similar changes in excretion of control subjects 3 and 4. The marked changes in creatinine excretion as related to weight in the control and experimental subjects are evident in the next to last column of table 2. The results suggest that the mechanisms concerned with the production and excretion of creatinine are relatively less affected in some subjects than in others.

The supplementation of vitamins was begun in February. Because of the variability of the weekly excretion and the long time required to obtain average results, the effect of individual supplements could not be studied. The welfare of the subjects had to be considered. The creatinine excretion of subjects 1 and 2 remained stationary during February and March when only thiamine had been given, and rose slightly during the succeeding months of April and May when other supplements were given. Subjects 5, 6, and 7 received

supplementations of casein along with the vitamins, beginning in February. Relatively little change in creatinine excretion was noted in this and the succeeding month. The physical condition of subjects 6 and 7 was not apparently improved and, therefore, it was decided to give additional supplements of 100 gm lean meat, 1 glass of milk, and 1 egg per day in April. Thus, the supplemented diet of these 2 subjects contained more protein than diet I; in fact, it contained 25 gm more of meat. As was to be expected, the creatinine excretion of subjects 6 and 7 rose, and in May was —8.7 and +1.3%, respectively, of the output of October (table 2). In spite of 2 months of complete supplementation, it was still below that of September (table 1). The work output of all of the experimental subjects, although improved, was still low in May after several months of complete supplementation.

The residual effects associated with dietary restrictions are further shown by a comparison of the results from September, diet I, and June, diet III (table 1). Subjects 3 and 4, who had received casein and vitamin supplements during 7 months of the preceding period of diet II, excreted 280 and 180 mg *more* of creatinine, or +13.1 and +9.8%, respectively, in June than in September. The previous diet, therefore, did not affect the response to diet III rich in meat and other dietary essentials.

On the other hand, subjects 1 and 2, who previously had received casein for 2 months and vitamins for 4 months, excreted 40 and 260 mg *less* (—2.3 and —12.1%), respectively, than in September. Subject 5, who had previously received both supplements for 4 months, excreted 150 mg *less* (—7.4%), than in September. In contrast to this, subjects 6 and 7, who had received supplements similar to subject 5, but had also received meat, milk and eggs in the previous 2 months, excreted only 60 and 130 mg more, or +2.8 and +7.2%.

The weight of all subjects had returned to approximately the same level in June as in September. The differences in creatinine excretion, therefore, cannot be ascribed to dif-

ferences in weight. They cannot be explained entirely on the basis of the creatine or creatinine content of the diets, nor are they directly related to the protein content, which, as far as is known, is the most important source of nitrogen compounds for the synthesis of creatine and, presumably, also of creatinine. It is not likely that the differences were due to storage of creatine or creatinine because the period of 2 (subjects 1 and 2) to 4 (subjects 5, 6, and 7) months of protein supplementation before June was longer than the time usually considered necessary for nitrogen equilibrium.

Creatine determinations (by difference) were made toward the end of January, the fourth month of diet II, before supplementation was begun. The calculated excretions per day were as follows in the order of subjects 3, 4, 1, 2, 5, 6, and 7: 10, none, none, 80, 60, none, 20 mg.

DISCUSSION

The experimental conditions used in this study differed somewhat from those employed in previous studies on the factors concerned with the excretion of creatinine. All subjects received the same weighed quantities of food. The quantity of protein ingested, therefore, was the same in all subjects, regardless of weight. The restricted diet contained a considerable quantity of corn meal. The diets were administered over a long enough period to assure equilibrium and to permit observations on the variation in the excretion of creatinine. The subjects received an average of 3200 cal. per day, which was greater than had been given in most previous investigations. The diets were monotonous; each subject expected and looked for symptoms of deficiency; none of the subjects was aware of the experimental details or the results until the experiment was completed. At least once on each of 6 days each week, exercises were performed to the point of exhaustion which led to a high lactic acid content of the blood.

The factors of physical and emotional stress, which perhaps were greater in this long experiment than in previous experiments with laboratory personnel or students, should be con-

sidered in an evaluation of the results. These factors may have affected the nervous and hormonal mechanisms which exert some control not only on chemical reactions in the body tissues, but also on renal excretion. To some extent, they may have been responsible for the apparently periodic variations of the creatinine excretion which was observed in this experiment during the normal period as well as in the period of dietary restriction. However, these factors and others associated with loss of weight do not account for the gradually decreasing average rate of creatinine excretion which was observed during the period of dietary restriction, and which persisted for several months after supplementation of protein and B-complex vitamins.

Our results lend support to the theories of Folin and Shaffer mentioned in the introduction concerning the relation between the creatinine excretion and the physical and nutritional state of the individual.

SUMMARY AND CONCLUSIONS

Seven men, ages 22 to 27 years, were subjected to a uniform regimen of physical exercises and tests over a period of 50 weeks, during which the variations of creatinine excretion, body weight, and physical performance were studied. The diets were as follows: a weighed, creatinine-containing normal diet I, containing 3170 cal., 70 gm protein and adequate quantities of minerals and vitamins, during 11 weeks; a weighed, essentially creatinine-free diet II, containing 3300 cal., 45 gm protein, greatly restricted quantities of B-complex vitamins, but otherwise adequate, over a period of 36 weeks — divided into equal periods of restriction and subsequent supplementations of thiamine, riboflavin, niacin, biotin, pteroylglutamic acid, pyridoxine, pantothenic acid and 40 gm casein; approximately equal quantities of a rehabilitation diet III, containing 250 to 350 gm of meat, during the last 3 weeks. After the fifth week of diet II, all supplements were given to 2 subjects who thus served as "controls" for a period of 7 months.

Creatinine was determined in composite samples collected over 4 days each week.

The creatinine excretion varied somewhat from week to week, often in a rhythmic manner, independent of the small changes of body weight in each subject. The maximum variations of the creatinine coefficient were of the order of $\pm 5\%$ of each mean from all subjects during 9 weeks of diet I, and $\pm 3\%$ of the mean from each of the control subjects during the first 9 weeks of diet II. These variations were minimized when the monthly averages were compared; the creatinine excretion then paralleled the rise or fall of body weight. During the first 5 weeks of diet II, the average coefficients of 6 subjects, weighing 62.0 to 77.0 kg, agreed within -1.5 to $+1.6$ mg, or -6.1 to $+6.5\%$ of the mean of 24.7 mg per kilogram per day; in the case of the seventh subject, who was fat and weighed 83.1 kg, the average coefficient was 22.2 mg. The average coefficients were in the same relative order of subjects with both diets I and II.

Administration of diet II to 5 subjects resulted in gradually decreasing average creatinine coefficients and physical performance which were paralleled to some extent by development of early clinical signs and symptoms of dietary deficiency. In 2 subjects, the administration of thiamine did not increase the average creatinine excretion or the coefficient within 2 months. Complete supplementation did not immediately increase the average creatinine excretion or the coefficient, nor did it rapidly improve the physical performance. In 2 subjects, the creatinine excretion and the coefficient were not raised to that of diet I when meat, milk and 1 egg were given in addition to all supplements (a total of about 110 gm protein) during the last 2 months of diet II. The effect of the previous dietary regimen was still evident during the period of the rehabilitation diet III, both by comparison of the results with those from the control subjects and by comparison of the individual data at the end of diet period II with those of diet period I.

Therefore, dietary restrictions with respect to protein and B-complex vitamins resulting in the development of deficiency symptoms may be reflected in metabolic changes associated with the formation and excretion of creatinine. These changes may persist, and are not rapidly abolished during subsequent supplementation.

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STEPHEN MOULTON BABCOCK

1843–1931

STEPHEN MOULTON BABCOCK

(October 22, 1843 – July 2, 1931)

With this first number of volume thirty-seven a new picture of a distinguished scientist graces the front cover of the Journal, that of Stephen Moulton Babcock. He was one of our American pioneers in agricultural and nutritional biochemistry. The photograph from which the picture on the cover was taken serves as a frontispiece for this volume.

Dr. S. M. Babcock was born at Bridgewater, New York, October 22, 1843, and died in Madison, Wisconsin, July 2, 1931. On October 27, 1896, he was married to May Crandall, but no children were born of this union. His father was Pelig Babcock and his mother Cornelia Scott.

He took his A.B. degree at Tufts College in 1866 and in 1901 received an honorary LL.D. from the same institution. In 1879 he received his Doctor's degree at the University of Göttingen, Germany. From 1875 to 1877 and from 1881 to 1882 he was instructor in chemistry at Cornell University and from 1882 to 1888 chemist at the Agricultural Experiment Station in Geneva, New York. In 1888 he became Professor of Agricultural Chemistry at the University of Wisconsin and Chief Chemist of the Wisconsin Agricultural Experiment Station. He became Assistant Director of that institution in 1889.

Many honors came to Dr. Babcock. He received a bronze medal from the Wisconsin Legislature in 1899; Grand Prize of the Paris Exposition in 1900; Grand Prize from the St. Louis Exposition in 1904, a silver medal from Stockholm in 1908; and the Capper Award for distinguished service to American agriculture in 1930.

Dr. Babcock's grasp of science was thorough, for he brought to his problems a deep understanding of mathematics, physics, and chemistry.

In the popular mind Dr. Babcock will be remembered longest for his invention of the milk fat test which bears his name. This was done in 1890 when he was 47 years of age. He has told me many times that his real contribution to the development of the milk fat test lay in his introducing the centrifuge as a part of the test, thereby shortening the time of operation. Here again his fundamental knowledge of physics stood him in good stead. Probably no contribution to agriculture by a scientist has helped more to gain the respect and confidence of farmers for agricultural experiment stations than the Babcock test. It made possible payment for milk on an equitable and impartial basis, and removed in a single stroke the obstacle that had so long stood in the way of utilizing factory methods in the manufacture of butter and cheese. The Babcock test permitted the accurate and easy determination of the fat production of individual cows, which made possible advanced registry, testing, and dairy herd improvement associations.

This inventive genius also developed a viscosimeter, the principle of which is the basis of the modern viscosimeter. Another important piece of work contributed by Dr. Babcock was his solution of the problem of metabolic water, a fundamental question in plant and animal physiology. This research is now considered a classic by plant physiologists and to animal physiologists it explains how a clothes moth can live on dry clothes and produce larva containing 75% water. It was published in Research Bulletin No. 22 of the Wisconsin Agricultural Experiment Station, March, 1912.

The present century has seen a great development in our knowledge of animal nutrition. Dr. Babcock's contribution to that development is not generally known. He lighted the torch for others to carry on. Dr. Paul de Kruif, author of Microbe Hunters, tells that story in his book Hunger Fighters. When

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More than 20 years of Dr. Babcock's later scientific career were spent in fundamental work in the field of physics, especially on the constitution of matter. Even in his earlier years he had given much thought to this subject and had formulated in his own mind an hypothesis that departed radically from the commonly accepted ideas concerning the relation of matter and energy. About 1896 he began a series

Dr. Babcock's grasp of science was thorough, for he brought to his problems a deep understanding of mathematics, physics, and chemistry.

In the popular mind Dr. Babcock will be remembered longest for his invention of the milk fat test which bears his name. This was done in 1890 when he was 47 years of age. He has told me many times that his real contribution to the development of the milk fat test lay in his introducing the centrifuge as a part of the test, thereby shortening the time of operation. Here again his fundamental knowledge of physics stood him in good stead. Probably no contribution to agriculture by a scientist has helped more to gain the respect and confidence of farmers for agricultural experiment stations than the Babcock test. It made possible payment for milk on an equitable and impartial basis, and removed in a single stroke the obstacle that had so long stood in the way of utilizing factory methods in the manufacture of butter and cheese. The Babcock test permitted the accurate and easy determination of the fat production of individual cows, which made possible advanced registry, testing, and dairy herd improvement associations.

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of experiments in which he weighed a large mass of water on a very delicately constructed balance to study the variations in the weight of the water in solid and liquid states. As was his custom, he constructed the entire apparatus with his own hands. The persistence with which he carried on these studies, even at the advanced age of 87, inspired the admiration of all his colleagues.

The later years of his life were spent in reducing his observations on matter and energy to written form, but he would not publish on the subject during his lifetime. After his death the manuscript which he left was critically examined by a number of prominent physicists and mathematicians, not only at Wisconsin but elsewhere. It was the general consensus that the work was more qualitative than quantitative and that the conclusions drawn were not sufficiently definite to warrant publication. Some expressed the opinion that many of the basic principles now recognized in the new science of physics were foreshadowed in the bold postulates that Babcock set forth in these early studies.

Dr. Babcock left no long list of contributions to scientific literature, for he was as sparing a writer as a speaker. For the most part he played a lone hand. The freshness of his ideas came from long brooding over a subject. His inquisitive mind was always subjecting the observed fact to more crucial experimentation.

Modesty and simplicity in practically everything were characteristic of Dr. Babcock. He was a joyous comrade, a friend beloved beyond measure. He pursued the most painstaking research as if he were playing a game. He brought laughter into the laboratory for there was about him that deceptively careless air which creative spirits have as they go about their business. He brought to his tasks that gaiety of spirit which authentic greatness can afford.

Wisconsin's President Frank said of him:

"This grand old doctor of science was himself greater than anything he did, and thus he gives to us, the legatees

of his spirit, a goal toward which to point the education and the science of our time.

"This merry man of many years was made of the stuff that gives mankind its saints and martyrs. But he was a saint without seriousness and he could have gone to martyrdom without a murmur of self pity, as part of the day's work. For his was a casual greatness."

E. B. HART

CORNEAL CHANGES IN THE RAT WITH DEFICIENCIES OF PANTOTHENIC ACID AND OF PYRIDOXINE[1]

LESTER L. BOWLES, W. KNOWLTON HALL, V. P. SYDENSTRICKER AND CHARLES W. HOCK

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NINE FIGURES

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With deficiencies of any of a number of essential nutrients which are important constituents of the corneal tissue, vascularization and other tissue changes may occur. Changes of this type have been observed in deficiencies of protein or of any of the indispensable amino acids (Sydenstricker et al., '47). Berg and coworkers ('47) suggested that with methionine deficiency the nourishment of the avascular corneal tissue becomes inadequate and an attempt is made by the tissue to compensate for the inadequacy by the development of capillaries to bring additional nourishment. This explanation might well apply to all nutritional deficiencies where such changes appear.

Wolbach and Howe ('25) described vascularization of the cornea in rats deficient in vitamin A, but regarded this as a phenomenon secondary to the hyperkeratinization of the corneal epithelium. Bessey and Wolbach ('39), on reviewing the material used in the previous study, were disposed to discard inflammation as an explanation of this vascularization. After a careful study of the corneal changes in riboflavin deficiency Bessey and Wolbach concluded that "vasculariza-

[1] A preliminary report of this work has been published (Bowles, '47).

tion of the cornea of the rat in the absence of antecedent pathology is probably a specific and most reliable criterion of riboflavin deficiency." The vascularization was accompanied in the advanced stages by leucocytosis and marked changes of the corneal epithelium. Their "control rats subjected to the following conditions did not develop corneal vascularization: B_1 deficiency, B_6 deficiency, fasting, old age, maintenance on the experimental diet plus riboflavin." György ('42) reports seeing no corneal vascularization in rats fed a diet lacking B_6. Singal, Sydenstricker and Littlejohn ('47) observed no corneal abnormalities in rats deficient in nicotinic acid.

It appears probable that there are unknown factors concerned in the prevention of corneal vascularization. György ('42) states that "keratitis and vascularization of the cornea were frequently encountered in rats that received vitamin B_1, riboflavin, vitamin B_6 and pantothenic acid as supplements to their vitamin-B-free diet." There is evidence that unknown factors are also involved in preventing corneal vascularization in man. Machella and McDonald ('43) studied a series of 20 cases where the patients appeared to have the symptoms of ariboflavinosis including, in some instances, corneal vascularization. However, the patients did not improve on administration of riboflavin. In some of 4,000 R.A.F. personnel studied by Lyle, McRae and Gardiner ('44) the corneal vascularization observed seemed to be influenced more by other factors present in fruits or vegetables than by riboflavin. These data led to a consideration of the possibility that corneal vascularization might result from deficiencies of B vitamins other than riboflavin.

EXPERIMENTAL

Pantothenic acid deficiency

In an attempt to produce corneal vascularization in pantothenic acid deficiency, 27 Wistar strain rats of various ages from 7 litters were placed on a pantothenic acid-deficient diet, as shown in table 1. The diet consisted of "vitamin-free"

TABLE 1

Corneal vascularization and growth of pantothenic acid-deficient and of control rats

LITTER NO.	AGE WHEN PLACED ON DIET	RATS ON DEFICIENT DIET	RATS ON CONTROL DIET	RATS WITH CORNEAL VASCULARIZATION	TIME OF [1] APPEARANCE	AVERAGE WEIGHT CHANGE
	days				*days on diet*	*gm*
1	22	5	0	0		+ 10
2	41	5	0	3	59 (43–74)	+ 43
3	50	6	0	4	71 (59–82)	— 3
4	52	3		3	61 (51–80)	+ 32
	52		3	0		+ 122
5	61	3		0		+ 2
	61		3	0		+ 95
6	61	3		1	41	— 14
	61		3	0		+ 125
7	61	2		2	102 (98–105)	— 36
	61		1	0		+ 111
Summary	22–61	27		13	68 (41–82)	+ 9
	52–61		10	0		+ 114

[1] The figures given are mean values and the range. Since biomicroscopic examinations were made at weekly intervals, the actual time of appearance of vascularization might be from one to 6 days earlier than these figures indicate.

casein [2] 20 gm, salt mixture [3] 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, pyridoxine hydrochloride 0.4 mg and sufficient sucrose to make 100 gm. Ten control rats from the same litters were placed on a control diet which was identical with the deficient diet except that it contained 2 mg of calcium pantothenate in each 100 gm. The care and treatment of the animals were as described in an earlier paper (Bowles et al., '46).

[2] Labco.

[3] The salt mixture was the same as that used by McKibben, Madden, Black and Elvehjem ('39).

Weekly biomicroscopic examination of the eyes of the first litter of rats which was placed on the diet when 22 days of age failed to show any significant change up to the time of death. These rats lived for 43, 48, 50, 76 and 89 days, respectively, on the deficient diet.

Thirteen of the 22 older rats on the deficient diet developed definite corneal vascularization, while 5 of the 22 died before sufficient time had elapsed for corneal vascularization to appear. The corneal capillaries which developed in rats fed the deficient diet were numerous and large with prominent anastomoses as contrasted with the finer network of vessels seen in riboflavin deficiency. The appearance of the capillaries in the cornea was preceded by a thickening of the cornea and by a diffuse or nebular opacity of slight degree. The opacity increased until at the time of death the cornea was nearly opaque and was frequently ulcerated (fig. 4). Figures 1 to 5 show varying degrees of these corneal changes. The patterns of development of the capillaries were similar to those previously described as occurring in other deficiencies (Bowles et al., '46). The corneal vascularization as seen with the biomicroscope and in the injected corneas is somewhat similar to that observed in early vitamin A deficiency (Bowles et al., '46) and in deficiencies of isoleucine or valine (Sydenstricker and others, '47).

Three of the rats with varying degrees of corneal changes were placed on the control diet and, in addition, were given 1 mg per day of calcium pantothenate. Within a few days the cornea became clear and the vessels assumed a beaded appearance and gradually became bloodless, beginning at the distal end. The time required for the vessels to become nonfunctional varied from a few days to several weeks depending on their size. Careful examination of the cornea with the biomicroscope and with the dissecting microscope then showed empty capillaries still present. In the limbic area short "stumps" filled with blood could still be seen (figs. 6 and 7).

None of the control rats showed any significant corneal changes.

In general, the rats on the deficient diet gained weight for a time and then lost weight at an accelerating rate for a period preceding death. The over-all weight changes during the course of the experiment are shown in table 1.

Pyridoxine deficiency

In order to study the ocular changes in pyridoxine deficiency, rats from 8 litters were placed on a pyridoxine-deficient diet [4] or on a control diet,[5] as shown in table 2. The techniques used and the details of the care of the animals in this study were described by Bowles and coworkers ('46). Weekly biomicroscopic examinations for ocular changes were made.

Up to the time of death of the rats placed on the deficient diet when 22 days of age no ocular changes had been observed. This is in accord with the observations of Bessey and Wolbach ('39) and of György ('42). The litter of rats first fed the deficient diet when 65 days of age developed no very marked symptoms of the deficiency and no ocular changes could be seen. Eleven rats of the 23 which were placed on the deficient diet when 45–57 days of age developed definite corneal vascularization.

The corneal capillaries were of a heavy branched type and their appearance was associated with a moderate opacity of the cornea (figs. 8 and 9). The corneal changes were not as extensive as in pantothenic acid or riboflavin deficiencies and the animals usually died soon after the corneal changes appeared.

In two animals with corneal vascularization which were changed to the control diet, a regression of the vascularization resulted similar to that described above for pantothenic acid deficiency.

[4] The pyridoxine-deficient diet consisted of vitamin-free casein (Labco) 20 gm, salt mixture (as used by McKibben et al., '39) 4 gm, cottonseed oil 3 gm, cod liver oil 2 gm, choline chloride 0.2 gm, thiamine hydrochloride 0.4 mg, riboflavin 1.6 mg, calcium pantothenate 2.0 mg and sufficient sucrose to make 100 gm. The control diet contained, in addition, 0.4 mg of pyridoxine hydrochloride.

[5] See footnote 4.

One of the 20 patients with symptoms of nutritional deficiency studied by Machella and McDonald ('43) improved after administration of pyridoxine. However, these investiga-

TABLE 2

Corneal vascularization and growth of pyridoxine-deficient and of control rats

LITTER NO.	AGE WHEN PLACED ON DIET	RATS ON DEFICIENT DIET	RATS ON CONTROL DIET	RATS WITH CORNEAL VASCULARIZATION	TIME [1] OF APPEARANCE	AVE. WT. CHANGE
	days				*days on diet*	*gm*
1	22	6	0	0		+ 7
2	45	4		1	88	+ 13
	45		2	0		+ 193
3	48	3		2	56 (56–56)	+ 15
	48		2	0		+ 125
4	53	3		0		+ 24
	53		3	0		+ 204
5	54	6	0	4	69 (43–78)	+ 26
6	55	4		2	87 (65–108)	+ 63
	55		3	0		+ 73
7	57	3		2	115 (108–122)	+ 83
	57		1	0		+ 92
8	65	6	0	0		+ 58
Summary	22–65	35		11	80 (43–122)	+ 35
	45–57		11	0		+ 142

[1] The values given are the mean values and the range. Since biomicroscopic examinations were made at weekly intervals, the actual time of appearance of vascularization might be from one to 6 days earlier than these values indicate.

TABLE 3

Corneal changes observed in rats in various vitamin deficiencies [1]

DEFICIENCY	VASCULARIZATION	OPACITY	SUPERFICIAL ULCERATION
Vitamin A	++++	++++	++++
Riboflavin	+++	++++	
Pantothenic acid	+++	+++	++
Pyridoxine	++	++	

[1] The corneal changes summarized here were described by Bowles et al. ('46) and in the present paper.

tors did not report the observation of any ocular changes in this particular patient.

DISCUSSION

Deficiencies of any of 4 vitamins may result in corneal changes in the rat. The extent of these changes as observed in this and a previous study is shown in table 3. This may be compared with a similar table in which Hall and coworkers ('48) indicate the degree of corneal and lenticular change in protein and amino acid deficiencies. Corneal changes of this type are not invariably observed in all nutritional deficiencies but presumably result from deficiencies of nutrients which are of primary importance in the structure and metabolism of the cornea.

SUMMARY

1. Heavy vascularization, thickening and opacity of the cornea were observed in 13 of 22 rats which were placed on a pantothenate-deficient diet when from 41 to 61 days of age.

2. Similar but less marked changes were seen in 11 of 23 rats placed on a pyridoxine-deficient diet when 45 to 57 days of age.

3. The changes observed were similar to those observed in early vitamin A deficiency and in deficiencies of isoleucine and of valine.

ACKNOWLEDGMENT

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PLATES

PLATE 1

EXPLANATION OF FIGURES

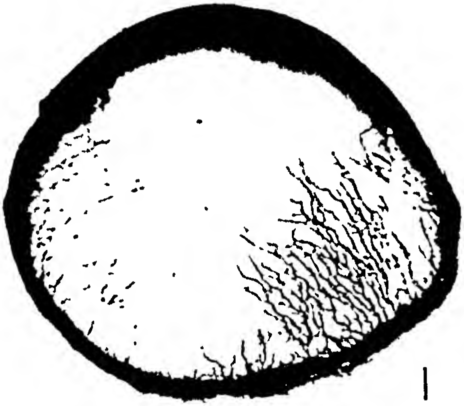
(All 18 × reduced approximately one-third)

Oblique views of India ink-injected corneas of rats fed a pantothenate-deficient diet.

1 and 2 Both corneas of rat from litter no. 4, table 1. Corneal vascularization observed after 51 days; rat killed after 76 days on the diet.

3 and 4 Both corneas of rat from litter no. 3, table 1. Corneal vascularization observed after 59 days; rat killed after 104 days on the diet.

5 Cornea of rat from litter no. 3, table 1. Corneal vascularization observed after 80 days; rat killed after 93 days on the diet.



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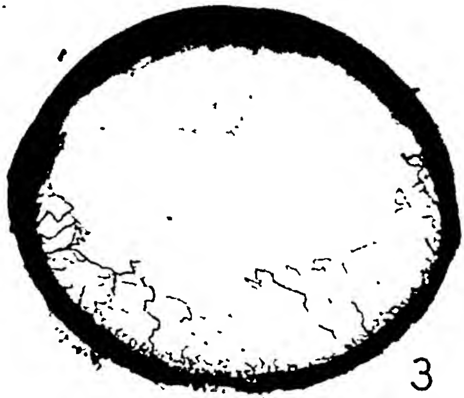

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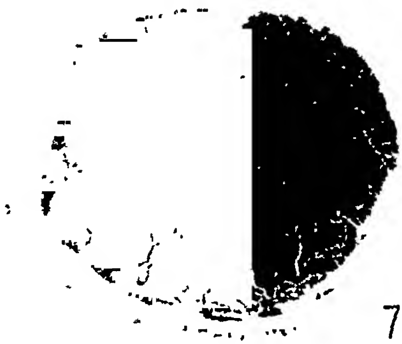

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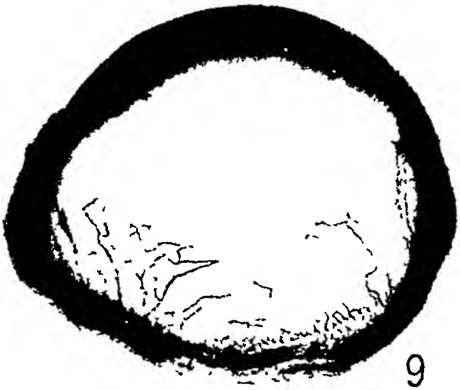

9

(All 18 × reduced approximately one-third)

Oblique views of India ink-injected corneas from 4 rats.

6 Pantothenate-deficient diet 87 days, then control diet 19 days. Corneal vascularization observed after 74 days. (From litter no. 2, table 1).

7 Pantothenate-deficient diet 97 days, then control diet 22 days. Corneal vascularization observed after 60 days. (From litter no. 2, table 1.)

8 and 9 Pyridoxine-deficient diet 171 and 96 days, respectively. Corneal vascularization observed after 78 days. (Both from litter no. 5, table 2.)

MOUSE GROWTH ASSAY PROCEDURES FOR THE "ANIMAL PROTEIN FACTOR"[1]

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FIVE FIGURES

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It has been well established that there is associated with animal protein materials an unidentified factor, or factors, not present in yeast or the major seed protein concentrates that is essential for normal growth and may also be necessary for the maintenance of life. The existence of such a factor has frequently been suggested by many workers. However, differences in the composition of the basal diets and in the degree of depletion of the test animals employed by different investigators have resulted in considerable confusion, not only with regard to the number of factors involved but also with regard to the nature of the deficiency symptoms to be expected.

This investigation was designed to develop a convenient assay procedure for the "animal protein factor" and to study, in part, its role in animal nutrition.

EXPERIMENTAL METHOD

During the war years the stock diet of the Sharp and Dohme mouse colony consisted almost entirely of a mixture of cereal grains and vegetables. Weanling mice taken from the colony

[1] A preliminary report of this study was presented before the American Chemical Society, Chicago, Illinois, April 19, 1948.

at this time showed a marked increase in growth rate when liver was added to basal diets containing either yeast or a mixture of synthetic B vitamins or both (Bosshardt et al., '45). Following the war, when meat scraps and dry skim milk were again used in the colony stock diet, this difference no longer could be obtained. The growth of the mice receiving liver was not significantly greater than that of those receiving the unsupplemented basal diet. This suggested a storage of the "animal protein factor" in the weanling mice sufficient to meet requirements for the duration of the test period.

TABLE 1

The effect of depletion of the "animal protein factor" on reproduction in the mouse

LITTER NO.	NO. FEMALES	NO. YOUNG WEANED	NO. YOUNG WEANED PER LITTER
1	35	247	7.1
2	30	199	6.8
3	28	117	4.2
4	26	0	0

Thirty-five pregnant female mice that were obtained from the Sharp and Dohme colony three to 5 days before parturition were placed on an animal protein-free diet. The composition of this diet was: 10% wheat germ middlings, 40% of a well heated soy flour, 18% hydrogenated cottonseed oil,[2] 3% cod liver oil, 7% dried yeast,[3] 18% glucose,[4] and 4% salt mixture (Jones and Foster, '42). This diet supported normal growth with weanling mice taken from the stock colony. The adult female mice were maintained on this diet exclusively until 4 successive litters were obtained.

Table 1 shows the number of mice weaned (16 days of age) per litter throughout the study. This number decreased with each successive litter until the 4th litter, when none was

[2] Primex.

[3] Anheuser-Busch, strain G.

[4] Cerelose.

raised to weaning age. This was not due to a decrease in the number of mice born in the successive litters but rather to an increase in *post partum* mortality, with all the deaths occurring during the first three days after parturition. It was also noted that the death of young mice involved complete litters or else all young survived. It must be concluded that if this "all or none" type of mortality was due to a deficiency of the "animal protein factor" there were pronounced differences in the reserve stores among the female mice employed. Whether or not death of the young mice can be attributed to a lactation failure is debatable. Since some of the mice died with milk in their stomachs, it would appear that the mothers were secreting milk but that the milk was deficient in some nutrient, presumably the "animal protein factor," required by the young mice. In the light of the conclusions reached by Allgeier et al. ('48) the evidence would appear conclusive that the basal diet employed in this study was inadequate for normal reproductive cycles in the mouse.

When the mice of the first three series of litters were weaned the males were placed on two test diets. The first diet contained 30% alcohol-extracted casein,[5] 25% hydrogenated cottonseed oil, 2% corn oil,[6] 20% glucose, 4% salt mixture (Jones and Foster, '42), 2% cellulose,[7] and 17% white dextrin. The following supplements were added per 100 gm of this diet: alpha-tocopherol, 4 gm; vitamin A, 900 U.S.P. units; vitamin D, 180 U.S.P. units; 2-methyl-1,4 naphthoquinone diacetate, 1 mg; choline chloride, 200 mg; thiamine hydrochloride, 0.8 mg; riboflavin, 1.6 mg; pyridoxine hydrochloride 0.8 mg; niacin, 4.0 mg; calcium pantothenate, 4.4 mg; para-aminobenzoic acid, 4.0 mg; and inositol, 21.6 mg. The second diet was of the same composition except that 1% of a defatted, dehydrated whole liver powder[8] was added at the expense of an equivalent amount of white dextrin.

[5] G. B. I. vitamin test.

[6] Mazola.

[7] Cellu flour.

[8] VioBin.

The weight gains that were obtained during a 15-day period are shown in table 2. Whereas the growth of the mice receiving the liver was the same for each of the three litters, there was a steady decrease in the growth rates of the mice from the successive litters that were not fed liver. These results show a positive correlation between the degree of depletion of the female mice maintained on the all-vegetable diet as determined by reproduction efficiency and the growth rates of the mice from the successive litters fed a diet containing no "animal protein factor." The growth rates of the mice receiving liver show that liver contains a substance, or substances, that correct a deficiency of this factor.

TABLE 2

Average weight gains of mice depleted of the "animal protein factor" and fed diets with and without added liver

LITTER NO.	AVE. WT. GAIN WITH NO LIVER	AVE. WT. GAIN WITH 1% DRY LIVER	DIFFERENCE
	gm	*gm*	*gm*
1	6.05	10.98	4.93
2	2.20	10.43	8.23
3	0.90	10.40	9.50

One study was made to determine the amount of liver in the diet necessary for a maximum growth response. For this study male mice obtained from the first litters of depleted females were used. Two liver preparations, a defatted, dehydrated liver powder [9] and Wilson's 1:20 liver concentrate powder, were incorporated at varying levels into the basal diet previously described and fed ad libitum to groups of 8 male weanling mice. The weight gains that were obtained are shown in figure 1. The effects of the two liver preparations were found to be the same, with a maximum effect observed at levels ranging from 0.5 to 1.5% of the diet. A diminished effect was found with the defatted, dehydrated whole liver powder when the level in the diet was increased from 1.5%

[9] See footnote 8, p. 23.

to 2%. As a result of this experiment the standard positive control employed in all subsequent work has been a diet fed ad libitum and containing 1% of the defatted, dehydrated whole liver powder.

Although weanling mice obtained from depleted females would be ideal assay animals, their use was discontinued because of the difficulties involved in obtaining sufficient numbers of uniformly depleted mice at any one time.

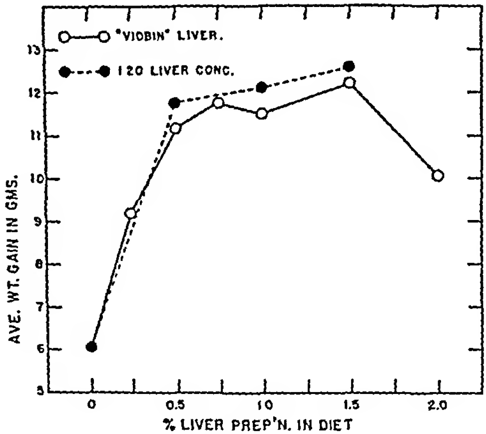

Fig. 1 The relationship between the 15-day weight gains of mice and the level of liver in the diet.

Ershoff ('47) has shown that liver contains a factor or factors distinct from all the known water soluble or fat soluble vitamins that completely counteracts the growth retardation resulting from the feeding of toxic amounts of thyroid-active materials. Preliminary experiments using an iodinated casein with thyroid activity [10] showed that similar results could be obtained with the mouse when the thyroid-active agent was incorporated into the basal diet at a level of 0.5%.

Table 3, the data of which are taken from the results of 8 successive studies, shows the 15-day growth responses of the negative and positive control groups. The mice in the negative

[10] Protamone. Cerophyl Laboratories.

control group received the "purified" basal diet previously described with the addition of 0.5% iodinated casein with thyroid activity,[11] and those in the positive control group received the same diet with the addition of 1% of a defatted, dehydrated, whole liver powder. The average increase in the growth rate attributable to the addition of liver was 62%.

It appeared that the use of the iodinated casein, or some other thyroid-active material, would make possible a relatively simple growth assay procedure. To determine whether the use

TABLE 3

The growth responses of mice fed 0.5% iodinated casein (Protamone) with and without added liver

AVE. WT. GAIN WITH NO LIVER	AVE. WT. GAIN WITH 1% DRY LIVER	DIFFERENCE	INCREASE WITH 1% DRY LIVER
gm	*gm*	*gm*	%
6.67	11.52	4.85	73
6.67	10.13	3.46	52
7.24	10.20	2.96	41
7.06	10.50	3.44	49
7.29	12.06	4.77	65
4.70	8.18	3.48	74
8.15	12.20	4.05	50
5.30	10.15	4.85	92
Ave. 6.64	10.62	3.98	62 ± 6.0 [1]

[1] Standard error $= \sqrt{\frac{\Sigma d^2}{N(N-1)}}$.

of depleted mice would be satisfactory as a test for the same activity as the use of mice fed the iodinated casein, assays using both types of assay animal were run on a series of preparations.

The samples that were used were prepared from three different sources, liver, pancreas and crude casein, and were chosen so as to give a range of potencies. The preparations were incorporated into the basal diets at identical levels and were fed to groups of 7 male weanling mice for 15 days. The depleted mice used were obtained from the second litters of

[11] See footnote 10, p. 25.

the depleted females. With each series a negative control group fed no liver and a positive control group fed 1% dehydrated, defatted liver were included. The potencies of the preparations were determined on a percentage basis, the differences in weight gains between the negative control mice and the mice receiving the preparation being compared with the differences between the negative and positive control groups. The results are shown in figure 2. A correlation coefficient of 0.89 was obtained between the two assays. Since these

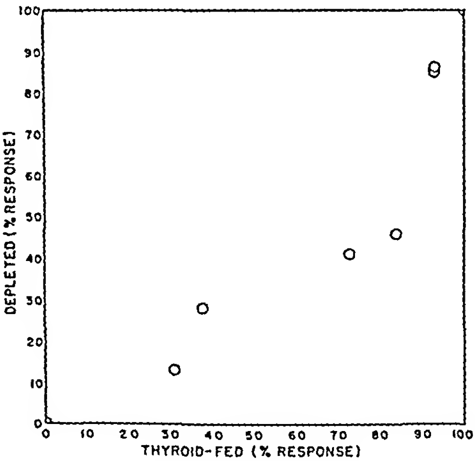

Fig. 2 A comparison of the responses of depleted mice and those fed iodinated casein (Protamone) when given "animal protein factor" preparations with varying potencies.

results suggested that the same activity was being measured by the two assay procedures, the use of mice fed iodinated casein appeared to be the better method because of its simplicity.

The growth retardation that was obtained by the addition of an iodinated casein with thyroid activity to a basal diet containing no source of the "animal protein factor" could not be obtained when a desiccated[12] or a defatted and dehydrated thyroid tissue[13] was used. The thyroid tissue may

[12] Armour and Co.

[13] See footnote 8, p. 23.

contain sufficient "animal protein factor" to counteract the growth retardation of mice attributable to thyroid activity. Another possibility is that mice and rats differ in their sus-

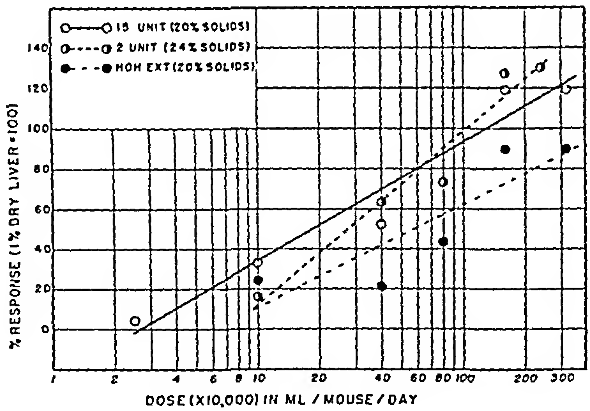

Fig. 3 The growth responses of mice fed the three liver preparations.

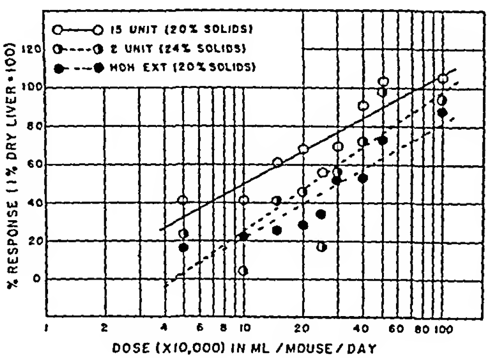

Fig. 4 The growth responses of mice given the three liver preparations by intraperitoneal injection.

ceptibility to the growth depressing effects of desiccated thyroid.

Using the mice fed iodinated casein, the relative potencies of three liver preparations were determined. These were: (1) a 15 U.S.P. unit per ml injectable liver extract; (2) a 2 U.S.P. unit per ml injectable liver extract; and (3) a water

extract of a defatted, dehydrated liver concentrated *in vacuo* to the same solid content as the two injectable extracts.

The results obtained with the oral feeding of the liver preparations for a 15-day period are shown in figure 3. The 15-unit liver extract was found to be about three times as potent as the water extract, with the two unit extract intermediate in effect.

The comparative potencies of the same three liver preparations were also determined when they were administered

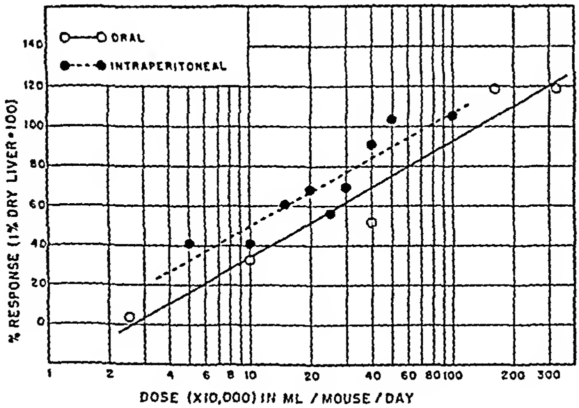

Fig. 5 A comparison of the growth responses of mice receiving a 15-unit liver extract orally and by intraperitoneal injection.

by intraperitoneal injection. The total dose for the 15-day period was given in two equal injections, one on the first day and the second on the 7th day of the period. The results, shown in figure 4, indicated that the same relative potencies resulted as were obtained by oral feeding. However, as is shown in figure 5, intraperitoneal administration resulted in about twice the response that was obtained when the liver extracts were given orally.

DISCUSSION

Some early workers, employing the rat growth method for vitamin A assay, noted that the growth of rats was sub-

optimum when highly purified casein was used as the protein source. The results of Coward et al. ('29) and Guha ('31) suggested that yeast was inadequate as a source of the water soluble vitamins. An improvement in the growth rate of their rats was noted when their basal diets were supplemented with such materials as fresh milk, wheat embryo, an extract of wheat embryo, ox muscle, liver, egg, grass, lettuce, or alfalfa.

Mapson ('32, '33) carried out one of the most complete of the early studies and showed that an apparently adequate diet could be improved by the addition of liver. His studies demonstrated quite conclusively that certain animal protein sources, particularly liver, contained a water soluble substance not present in yeast that had a stimulating effect on the growth and lactation of rats. His failure to find an effect when wheat germ was added to his basal diet would suggest either that his factor, named "physin," was distinct from the factor proposed by Coward et al. ('29) or that the latter workers were dealing with a multiple deficiency. The data presented in the present report lend support to the first of these hypotheses, since unextracted wheat germ was used as a component of the animal protein-free depletion diet.

Troescher-Elam and Evans ('41) have shown that supplementation of a basal diet containing pure B vitamins and cod liver oil with liver or an extract of liver will improve the growth of male mice. Increasing the levels of casein, minerals, or B vitamins produced no effect. McIntire et al. ('43) reported that liver contains a substance necessary for the optimum growth of rats receiving the pure B vitamins. This finding was tentatively interpreted by Zucker and Zucker ('44) as indicating that the factor was associated with high quality proteins as an essential amino acid or as an impurity, since they could find no effect when the casein level in their basal diet was increased to 24% (N $\times$ 6.25).

White and Sayers ('42) observed a more rapid growth rate and a higher food intake with rats fed a diet containing a pancreas derivative as the protein source than when a similar diet containing casein was fed. Their basal diet contained yeast

as the source of vitamin B. It was shown, however, by Bosshardt et al. ('45) that this increased growth rate could be attributed to a water soluble factor present in the pancreas preparation. Similar results were obtained when an extract of liver was added to the basal diet containing casein as the protein source. Bosshardt et al. further showed that the effects of liver could be enhanced by increasing the level of protein in the diet.

Hartman, Dryden and Cary ('41), Hartman and Cary ('42) and Cary and Hartman ('47) have suggested that a factor essential for the optimum growth of rats is present in liver, beef muscle, milk products, and certain grasses. Their factor, nutrient X, is not present in yeast or whole wheat, nor is it identical with any of the known vitamins. Nutrient X is presumed to be relatively heat stable.

In a study of the effects of different grains on the growth of chicks fed a soybean meal diet Whitson et al. ('45) found that wheat was superior in this respect to corn or barley. The addition of 3% sardine meal or 8% dried cow manure to the corn or barley diets appeared to supply these rations with the factor or factors responsible for the superiority of wheat over corn. These results would confirm those of Coward et al. ('29) to the effect that wheat contains an unidentified growth factor. Recently Novak and Hauge ('48) have obtained evidence that distillers' dried solubles contains a heat stable factor essential for rat growth. Prior to the work of Whitson et al., Hammond ('42, '44) showed that cow manure contains a growth promoting factor for chicks. This was confirmed by Whitson et al. ('45) and by Rubin and Bird ('46). The latter workers also demonstrated an equivalent growth promoting effect produced in chicks with Wilson's liver fraction L, which would suggest that the factor or factors in cow manure and liver are identical.

Jaffe ('46) and Jaffe and Elvehjem ('47) have shown the presence in liver of a heat labile factor which enhances the growth of rats. They could not produce any similar effect with a butanol soluble fraction of liver, which would appear to

differentiate this factor from that studied by Bosshardt et al. ('46). Jaffe and Elvehjem ('47) and Robblee et al. ('48) also have shown that fish press water (fish solubles) contains a growth factor for rats similar in many properties to the heat labile factor of Jaffe.

The results of Ershoff ('47) and Ershoff and McWilliams ('48) showed that the feeding of liver would completely counteract the retardation of growth in rats fed toxic amounts of thyroid and ovarian development in immature rats fed massive doses of alpha-estradiol. Wheat germ was without effect, whereas a slight effect was noted with yeast. Whole liver and yeast were both effective in prolonging the survival of the rats fed the thyroid-containing diets.

In a recent study Zucker and Zucker ('48) have described a factor, zoopherin, that is present in liver, crude casein, fish solubles, and cow manure and that is essential for the growth of rats fed yeast-containing plant rations. These authors suggested that zoopherin may be identical with the nutrient X of Hartman, Dryden and Cary and Rubin and Bird's cow manure factor.

The lack of complete agreement among the workers in the field of unidentified nutrients would suggest that more than one factor is involved. Other variables contributing to the confusion may be differences in the basal diets employed and differences in the degrees of depletion of the test animals used.

The evidence appears to indicate that liver contains a multiplicity of unidentified growth factors. At least one of these factors is present in wheat and another, or the same factor, is present in yeast. Liver may contain at least two factors not present in yeast, one of which is heat stable and the other heat labile. This, however, needs to be investigated further since preliminary studies in this laboratory (unpublished) indicate that the stability to heat may be dependent upon the state of purification of the materials tested.

Vitamin B_{12} has recently been isolated in pure form by Rickes et al. ('48) and Smith ('48). This material has been

shown by Ott et al. ('48) to possess growth-promoting properties for chicks similar to those shown by preparations of liver and fish solubles, suggesting the possibility that vitamin B_{12} may be identical with the "animal protein factor." Further work with vitamin B_{12} should help to clarify the present complications with regard to unidentified growth factors.

SUMMARY

Two mouse growth methods have been developed for the assay of the "animal protein factor." One method involves the use of growing mice born of mothers that were maintained on a diet free of this factor. The second method is based on the ability of the "animal protein factor" to counteract the growth retardation of mice that are fed thyroid-active materials.

The results of these studies showed that: (1) Liver contains a factor or factors essential for growth not present in yeast or wheat germ, nor identical with any known vitamin factor; (2) this factor counteracts the growth retardation of mice that are fed thyroid-active materials; (3) the "animal protein factor" may be transmitted from the mother to the young during gestation or lactation or both and may be stored by the animal for a considerable period of time; (4) a severe lack of the "animal protein factor" in the maternal diet and tissue stores results in a pronounced mortality of young mice one to three days *post partum*.

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INFLUENCE OF AGE AND OF VITAMIN A INTAKE ON THE STORAGE OF VITAMIN A IN THE LIVER OF THE RAT[1]

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Those who have had extensive experience in assaying for vitamin A by the biological method have frequently observed wide variations in the time required to deplete young rats of their body store of this vitamin. This difference in the time required for depletion usually varies with young rats from different colonies, from different genetic strains, and from the same breeding stock but subsisting on different rations. However, variations in the rate of depletion of young rats from the same breeding colony and fed the same rations are also observed. Such variations sometimes appear to be seasonal and are frequently attributed to variations in the vitamin A or provitamin A content of the breeding colony diet.

There are, however, variations in the time required to deplete young rats of their body reserves of vitamin A which cannot logically be attributed directly to genetic or dietary differences. These variations are usually associated with differences in the size and in the age of the test animals when placed on the vitamin A deficient diet. That such variations have been recognized in the past is attested by the fact that the U. S. Pharmacopoeia ('47) has prescribed definite age

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and weight limitations for the young rats used in official assays. While the probable effects of variations in age, in body weight, and in previous vitamin A intake on the rate of vitamin A depletion have been recognized, the extent to which these variables determine the time required to deplete the test animals has not been adequately studied. The present report relates to the effects of age and of previous vitamin A intake on the amount of this vitamin stored in the body of the rat.

Since ample evidence is at hand to show that the liver is the principle vitamin A storage tissue of the rat (Osborne and Mendel, '18; Sherman and Boynton, '25; Moore, '31; Baumann et al., '34; McCoord and Luce-Clausen, '34; and others), the vitamin A content of this organ has been considered a reliable index of the vitamin A reserve of this animal.

EXPERIMENTAL

The first phase of the investigation involved normal rats of various ages selected from our breeding colony. These rats were from breeding stock which had been reared and maintained through many generations on a complex breeding ration of our own formulation. Rats of both sexes, ranging in age from one to 749 days, were used. While Lemley et al. ('47) have shown that storage of vitamin A in the liver of the rat is not affected by sex differences, a special effort was made to determine the vitamin A content of livers from female rats which had previously weaned litters and which represented a wide range of reproductive ages. It was believed that these data would give some indication as to whether the progeny would readily respond to vitamin A depletion.

All animals except the unweaned young were weighed and sacrificed by decapitation 24 hours after their last feeding period. The abdominal cavity was opened at once and the entire liver removed, pressed between two sheets of heavy filter paper to remove blood and adhering body fluid, weighed and transferred immediately to an Erlenmeyer flask containing an appropriate volume of freshly prepared alcoholic

potassium hydroxide solution (10%), and then macerated by means of a blunt-end glass rod. The flask was attached to a reflux condenser and the contents digested on a hot water bath until the animal tissue had completely disintegrated. The digest was cooled, transferred to a separatory funnel, 100 ml of distilled water added, and the mixture extracted three times with 50 ml portions of peroxide-free diethyl ether. Previous experience had indicated that three extractions were sufficient to remove all of the vitamin A. The combined ether extract was washed free of alkali with distilled water, dried over anhydrous sodium sulfate, concentrated to an oily consistency through vacuum distillation, and the residue taken up in purified chloroform. The vitamin A content of the chloroform extract was determined by means of the Carr-Price procedure ('26) as modified by Dann and Evelyn ('38), using an Evelyn photoelectric colorimeter equipped with a no. 620 filter to measure the intensity of the color. At first the colorimeter was standardized with U.S.P. Reference Cod Liver Oil no. 3, but in the latter phases of the study the instrument was restandardized with the new U.S.P. Vitamin A Reference Standard (crystalline vitamin A acetate in cottonseed oil). All vitamin A values reported are calculated on the basis of the Vitamin A Acetate Standard. Individual determinations were run on all liver tissues except those from the one-day-old rats, in which cases the livers were pooled before weighing and saponification and the total vitamin A content of the unsaponifiable extract was determined. In all instances the vitamin A values reported are the average for all animals constituting the respective experimental groups.

A second phase of the investigation consisted of determining the relative amounts of vitamin A stored in the livers of rats as the result of feeding increasing daily doses of a natural ester concentrate of vitamin A (882,400 U.S.P. units per gram) under conditions comparable to those observed in the official U.S.P. biological assay. In this phase, a total of 27 experimental groups were used. The amount of vitamin A fed daily varied from 0.73 to 75,520 U.S.P. units. All of the

test animals were from our own breeding colony, produced under the usual breeding conditions and weaned at ages ranging from 21 to 24 days. All animals were caged in individual all-metal units during both the depletion and feeding periods. They were fed liberal quantities of a vitamin A deficient diet consisting of 18% purified casein, 4% salt mixture, 8% irradiated brewers' yeast, 5% hydrogenated vegetable oil, and 65% dextrinized corn starch. When the animals had become depleted of their body stores of vitamin A, they were assigned to experimental groups just as if they were to be used in an official vitamin A assay. Each rat was then fed daily a definite amount of the vitamin A concentrate after the concentrate had been properly diluted with refined cottonseed oil, so that the daily allotment of oily supplement was 0.1 ml in all instances. At the lower levels of vitamin A supplementation the vitamin was fed in individual fraction cups, but with the larger dosages (1,760 U.S.P. units daily and above) the supplement was placed directly in the rat's stomach by means of a special needle attached to a tuberculin syringe. The feeding of the supplement was continued for 28 days. The animals were then slaughtered and their livers removed, saponified and their vitamin A content determined in the manner previously described. The vitamin A content of the various livers was calculated in terms of the new U.S.P. standard and the values reported are average values for all animals constituting the respective experimental groups.

During the course of the previously described experiments other nutritional studies involving various diets were in progress in the laboratory. It was believed that a study of hepatic vitamin A storage under these experimental conditions would be of interest. Therefore, at the termination of the experiments the animals were sacrificed and the vitamin A content of their livers determined. Among the animals investigated were those which had previously received the Steenbock rachitogenic diet, the U.S.P. thiamine deficient diet, a breakfast-cereal diet, and the latter diet supplemented with milk, with vegetables, and with both milk and vegetables.

The pertinent data obtained in the course of these studies have been condensed and arranged in tabular form and are presented in tables 1 to 3, inclusive.

DISCUSSION

In the present studies it was of interest to find that the liver of the newborn rat contains only a limited amount of vitamin A (see table 1) and that the storage of the vitamin in that tissue did not increase greatly during the nursing period.

TABLE 1

Vitamin A content of livers of rats of various ages taken directly from the breeding colony

GROUP NO	NO OF RATS USED	SEX	AGE OF RATS	WEIGHT OF RATS (ave)	WEIGHT OF LIVERS (ave)	TOTAL UNITS FOUND (ave)	UNITS/GM LIVER (ave)	UNITS/GM BODY WEIGHT (ave)
			days	*gm*	*gm*	*U S P.*	*U S P.*	*U S.P.*
1	8	?	1	6 2	0.36	7	20	1.1
2	5	?	7	11.2	0.70	25	44	3.9
3	5	Males	16	30	1.98	105	54	3.5
4	4	Males	21	42	2.6	130	50	3.1
5	4	Males	37	89	5.9	360	61	4.0
6	4	Males	51	127	7.3	700	95	5.5
7	3	Males	73	212	10.1	1,860	184	8.8
8	1	Female	117	178	8.7	5,700	655	32.0
9	6	Males	170	361	12.2	22,470	1,862	63.0
10	3	Females	330	241	10.2	18,950	1,769	82.3
11	1	Female	334	202	9.5	16,190	1,704	77.8
12	1	Female	437	279	10.4	19,840	1,908	71.3
13	4	Females	564	281	10.4	18,440	1,762	65 9
14	4	Females	741	269	10.9	18,600	1,708	69.5
15	6	Males	749	421	14.5	28,400	1,865	68.5

This seems to indicate that the vitamin A present in the dam's milk is barely enough to provide for the active physiological processes associated with the rapid growth of the young. That the rate of metabolism is a factor in vitamin A storage in the rat is indicated by the fact that as a rule those weanling rats which consume the most food and which grow most rapidly during the early days of the vitamin A depletion period are invariably the animals which manifest deficiency symptoms

at the earliest date. However, the recent studies of Johnson and Baumann ('48) indicate that a 50% increase in growth rate is more conducive to low liver storage of vitamin A than is a three-fold increase in metabolic rate induced by feeding desiccated thyroid.

Livers of day-old rats were found to contain an average of 7 U.S.P. units of vitamin A per liver, or 20 units per gram of liver. The former value is in agreement with the value reported by Williamson ('48) for the vitamin A content of livers of normal unborn rats and hence may be indicative of the amount of vitamin A imparted to the young through placental circulation.

The livers from the 7-day-old rats appeared to have undergone a three-fold increase in their vitamin A content during the first week of postnatal life. However, owing to an increase in liver size the increase in vitamin A concentration was only two-fold during this period. The livers from other young rats slaughtered at 16 and 21 days of age, respectively, showed further increases in vitamin A deposition, but the increase in vitamin concentration per gram of rat liver or per gram of body weight during this period appeared rather insignificant. Perhaps this again reflects the vitamin content of rat's milk; it may indicate why the nursing young are readily depleted of their body stores of vitamin A. Livers from 37-day-old rats were found to contain almost three times as much vitamin A as livers from 21-day-old animals, and this increase in vitamin store was reflected in greater concentration of the vitamin per unit weight of liver and per gram of body weight. As the age of the rats increased further, the amount and the concentration of vitamin A found in the liver also increased rapidly. Livers from 51-day-old rats were found to contain twice as much vitamin A as did livers from 37-day-old rats, while livers from 73-day-old rats contained two and one-half times as much vitamin A as did livers from 51-day-old rats. This rapid increase in hepatic vitamin A with increase in age would seem to explain why it is extremely difficult to deplete the older animals.

Hepatic vitamin A continued to increase with age. The livers from 170-day-old male rats were found to contain more than 10 times as much of the vitamin as did the livers from 73-day-old rats, whereas the livers from 749-day-old males contained more than 15 times as much. Similarly, the hepatic vitamin A of female rats which had previously weaned litters and then undergone a 20-day rest period was found to increase so long as the body weight increased. The liver of a 117-day-old female weighing 178 gm was found to contain 5,700 U.S.P. units of vitamin A, whereas the livers from 330-day-old females (average weight, 241 gm) contained 18,950 units. However, further increases in the age of the females without significant increases in body weight did not result in any appreciable increase in liver storage of the vitamin. It was of particular interest to find that the hepatic vitamin A of both male and female rats reached its maximum concentration when the rats were approximately 170 days old. Although vitamin A storage increased beyond this age, the concentration in the liver did not increase. Since hepatic vitamin A values considerably greater than those found in the present studies have been observed, it is believed that the values herein reported do not represent saturation values but instead are probably optimum storage values for the dietary regime employed.

Livers from young rats which had been subjected to vitamin A depletion to the extent that they lost about 20% of their maximum body weight were found to contain no vitamin when the concentrates were examined by the antimony trichloride method (table 2). On the other hand, when the nonsaponifiable residues from similar livers were dissolved in isopropyl alcohol and the solutions examined at 328 mμ by means of the Beckman quartz spectrophotometer, a small but definite amount of absorption was invariably indicated. It was believed that this absorption was due to extraneous materials present in the nonsaponified fraction and not to vitamin A. However, spectrographic examination of concentrates from other rat livers known to contain vitamin A indicated a

TABLE 2

Relation of the vitamin A intake of the rat to the amount stored in the liver while subsisting on the U.S.P. vitamin A-deficient diet

ANIMAL GROUP NO.[1]	UNITS FED DAILY [2]	CHANGE IN WEIGHT [3] (AVE.)	TOTAL UNITS FOUND (AVE.)	UNITS/GM LIVER (AVE.)	UNITS/GM BODY WEIGHT (AVE.)	VITAMIN A RECOVERED (AVE.)
	U.S.P.	*gm*	*U.S.P.*	*U.S.P.*	*U.S.P.*	%
1	None [4]		0	0.0	0.00	..
2	0.73	+ 12	18	3.9	0.12	88
3	2.2	+ 67	34	4.1	0.17	55
3A [5]	2.2	+ 64	37	4.2	0.19	60
4	4.4	+ 69	52	5.1	0.26	42
5	7.4	+ 70	59	5.7	0.31	28
6	14.7	+ 74	61	5.7	0.31	15
7	22	+ 79	68	6.1	0.33	11
8	29	+ 80	71	6.5	0.34	9
9	44	+ 98	160	12.8	0.70	13
10	88	+ 85	340	28.8	1.62	14
11	176	+ 87	550	44.8	2.57	11
12	290	+ 76	1,070	87.7	4.8	13
13	590	+ 82	2,930	210	12.9	18
14	880	+ 76	3,900	290	17.6	16
15	1,760	+ 90	8,950	640	40.3	19
16	2,640	+ 77	9,360	730	45.2	13
17	3,520	+ 85	13,690	1,140	62.5	14
18	4,400	+ 72	62,820	5,510	310	51
19	6,600	+ 65	74,100	6,330	380	40
20	8,800	+ 71	92,580	8,050	450	38
21	17,600	+ 20	105,360	12,630	700	21
22	18,380	+ 20	92,210	9,510	630	18
23	26,400	+ 8	117,260	11,380	860	16
24	35,200	— 22	116,800	13,710	1,050	12
25	37,760	— 27	87,330	11,640	960	6
26	75,520	— 21	104,990	12,780	970	5

[1] Consisting of from 5 to 12 rats per group.

[2] Fed for a 28-day test period.

[3] Average weight of rats at end of depletion period was 128 gm.

[4] The animals of this group were maintained on the vitamin A-deficient diet for approximately 32 days or until they had lost, on an average, 28.4% of their maximum weight.

[5] The animals of this group were fed 3 mg of alpha-tocopherol daily during the 28-day test period as an additional supplement.

somewhat higher vitamin potency than did the results of the antimony trichloride method of assay.

When depleted rats were fed increasing doses of vitamin A ranging from 0.73 to 29 U.S.P. units per day over a 28-day period, there was a slight but measurable increase in the total amount of vitamin A stored in the livers, and the efficiency of storage, at the lower levels of intake, was found to be relatively high. On increasing the dosage to 44 U.S.P. units daily, the rats attained a maximum rate of growth and showed uniformly marked increases in hepatic vitamin A, as indicated both by total units and by concentrations. Further increasing the daily vitamin A intake in progressive stages from 44 through 8,800 U.S.P. units resulted in greatly increased amounts of hepatic vitamin A with each increment in intake. In fact, when the daily dosage ranged from 29 to 3,520 U.S.P. units, the hepatic vitamin A was found to approximately double for each two-fold increase in daily intake. However, over this range of vitamin intake the rates of growth of the animals constituting the several experimental groups were approximately the same. On increasing the vitamin A dosage from 17,600 to 75,520 U.S.P. units daily, in progressive amounts, there was no appreciable increase in hepatic vitamin A. In fact, the data indicate that somewhat less vitamin A was stored on the highest levels of intake (37,760 and 75,520 U.S.P. units daily) than on some of the lower levels of intake (26,400 and 35,200 U.S.P. units daily), but this condition is believed to be related to the depressed growth rate associated with the higher levels of vitamin intake. However, with these higher rates of intake the concentrations of vitamin A per gram of liver or per gram of body weight were in line with those obtained on the lower levels of intake. In connection with these storage studies it was of interest to observe that a previously depleted female rat, when fed 8,800 U.S.P. units of vitamin A daily for 28 days, retained sufficient vitamin to permit the production of three litters of young and the weaning of two litters, and to prolong the life of the female for 492 days.

In these studies it was noted that no improvement in growth rate resulted from feeding daily dosages of vitamin A in excess of 44 U.S.P. units, although the concentration of vitamin in the liver was far from optimum at that level of intake. Furthermore, it was noted that when the daily vitamin A intake was increased beyond 8,800 U.S.P. units daily, the growth rate was rather consistently depressed. The physiological complications arising from excessive intakes of vitamin A merit study and, as previously mentioned, the matter is already receiving consideration in this laboratory. The amount of vitamin A required to produce optimum growth in the present studies (44 U.S.P. units daily) is somewhat higher than that reported by Lewis et al. ('42); the difference may be due to the fact that the animals used in the present studies were depleted before being placed on test.

It has frequently been suggested that the vitamin E intake has an important bearing on vitamin A storage (Moore, '31; Davies and Moore, '41; and others). To determine whether hepatic vitamin A could be increased under the conditions prevailing in the present experiments by feeding a vitamin E supplement, a group of 11 depleted rats (Group 3A) was fed 3 mg alpha-tocopherol daily, along with 2.2 U.S.P. units of vitamin A, during the usual 28-day feeding period. The results show that there was no increase in either the growth rate or in the amount of vitamin A stored as the result of feeding the tocopherol supplement.

Another factor which appears to influence vitamin A storage is the duration of the period of vitamin A feeding. Glover et al. ('47) found that when moderate to extremely high dosages of vitamin A were administered in the form of a single dose to 6- to 9-month-old-rats and their livers examined from three to 14 days afterwards, rather inefficient liver storage of the vitamin had resulted (1 to 7%). On the other hand, Lemley et al. ('47), using a three-day feeding period, reported a more efficient storage of vitamin A than was observed in the present investigation except where the dosage ranged from 4,400 to 8,800 U.S.P. units daily. In this connection, it was of

interest to note that the most efficient hepatic vitamin A storage occurred when the vitamin A intake was approximately 4,000 units daily.

Foy and Morgareidge ('48), using a modification of the Guggenheim and Koch technic ('44), found that when partially depleted rats were fed daily doses of vitamin A ranging from 71 to 298 U.S.P. units for two successive days, and then slaughtered on the 4th day, from 13 to 38% of the vitamin could be recovered from the livers. According to the data of these authors, the amount of the vitamin stored in the liver of the rat depended on the intake, and the efficiency of the storage seemed to be greatest where the higher dosage was fed. On the other hand, according to our data, if somewhat comparable amounts of vitamin A (142 and 586 U.S.P. units) had been fed to fully depleted rats over a 28-day period, the efficiency of vitamin storage would have been greatest with the lower level of intake. Our findings show that the efficiency of vitamin A storage was lowest where the dosage was in the range of the minimum amount required to produce optimum growth, or where extremely high doses were fed. In these respects the data herein reported confirm those previously reported by Lemley et al. ('47). The present data are also in agreement with those of Lewis et al. ('41) in that maximum growth in the rat was obtained with a daily vitamin A intake ranging from 25 to 50 U.S.P. units, but the data do not confirm the findings of these authors that more than 10 units of vitamin A must be fed daily before any vitamin is stored in the liver.

An examination of the results obtained by assaying the livers of rats which had been maintained on several experimental diets for specific periods reveals some points of interest (table 3). Livers from 52-day-old rats which had subsisted on the Steenbock rachitogenic diet for 28 days contained approximately one-third as much vitamin A as did the livers from rats of comparable age taken directly from the breeding colony. Since the rats which had received the rachitogenic diet were subnormal in weight, part of this difference is accounted for by the difference in weight of the

2. The concentration of vitamin A in the liver of the nursing rat more than doubled during the first 7 days of postnatal life and then remained relatively constant during the remainder of the nursing period.

3. The amount and the concentration of vitamin A in the liver of the normal weanling rat increased rapidly throughout the period of rapid growth and reached a maximum concentration when the rat was about 170 days old. Beyond this age, additional vitamin A was stored but the concentration per gram of liver remained relatively constant.

4. The amount of vitamin A stored in the livers of depleted rats depended upon the intake, but no marked increase in concentration of hepatic vitamin A occurred until the intake was more than sufficient to promote optimum growth. Below this level of intake, increase in vitamin A storage as indicated by increased concentration was largely hidden by increase in weight of liver.

5. With vitamin A intakes in excess of that required for optimum growth, liver storage increased in proportion to the intake until a daily intake of 17,600 U.S.P. units was reached. Intakes of vitamin A in excess of this amount did not result consistently in additional vitamin A storage when fed under the conditions of these experiments.

6. Optimum growth was brought about in depleted rats when 44 U.S.P. units of vitamin A were fed daily, whereas daily dosages in excess of 8,800 U.S.P. units had a definite depressive effect on the growth rate.

The effects on liver storage of vitamin A in the rat resulting from ingestion of other types of experimental diets are presented and discussed.

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THE THIAMINE REQUIREMENT OF THE NORMAL INFANT[1]

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TWELVE FIGURES

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The problem of assessing vitamin requirements has been complicated by the discovery of a number of variable factors which may influence them. Vitamin requirements are conditioned by pathological processes which affect absorption or utilization or increase the need for particular factors. But even in the normal subject there are wide fluctuations in requirements, caused by the nature of the food, its effect on the bacterial synthesis of vitamins in the intestine, and its content of antivitamins. Alterations in the diet and bacteriostatic drugs are known to affect the synthesis of vitamins in the intestine. Vitamins present in the diet may be destroyed before ingestion by exposure, processing or cooking, and in the intestine they may be rendered unavailable by agents which compete with, combine with, adsorb or destroy them.

In the case of the infant many of these complicating factors do not enter the picture. The infant artificially fed along conventional lines receives a diet which is relatively constant in its proportion of calorigenic foodstuffs and in their source,

[1] The expenses of this study were defrayed in part by grants from the American Dairy Association, the Sugar Research Foundation, and Mead Johnson and Company

and antivitamins are not known to be present. Under these conditions it would seem possible to define vitamin requirements with reasonable accuracy and to determine what margin of safety our usual artificial feedings provide. The present report deals with an attempt to determine the minimum requirement of the normal infant for thiamine.

A minimum food requirement may be defined as that intake which will just protect against definite clinical symptoms or accepted laboratory criteria of deficiency. It was not deemed justifiable to deplete infants to the point of clinical deficiency, and laboratory criteria were therefore used as an endpoint. The selection of such criteria deserves some discussion since there is lack of agreement in regard to their merits. Thiamine deficiency has been measured by assays of thiamine in biopsied tissues, by determination of thiamine in serum, by the cocarboxylase level of the blood cells, by the pyruvate levels or the pyruvate: lactate ratio of the blood serum, and by various excretion procedures for measuring urinary thiamine. The biopsy technique, aside from obvious objections, is undesirable because of its inaccuracy and the paucity of "normal" standards. Pyruvate levels and pyruvate:lactate ratios are influenced by non-specific factors (exercise, excitement) which are difficult to control in infants and were therefore to be avoided. The analytical methods for blood thiamine and cocarboxylase measurement at the time our study was begun were not considered sufficiently reliable and accurate, and some urinary excretion procedure therefore seemed the best method for estimating the degree of deficiency.

Three types of urinary excretion tests are commonly employed: (1) the 24-hour urinary excretion test, (2) load tests, and (3) fasting excretion tests. These have been applied to thiamine itself and to its pyrimidine decomposition product variously known as "pyramine" or PAYF (pyrimidine activator of yeast fermentation), an inert metabolite. We employed the 24-hour excretion test as being the most suitable for our purpose and confined our observations to the

excretion of thiamine itself. Although a load test has certain advantages, particularly in evaluating well-developed clinical deficiencies, we did not use one because of the delay involved in reaching equilibrium after the introduction of a test dose. Ordinarily the 24-hour excretion test is objectionable in that a postprandial rise in excretion follows the ingestion of a thiamine-containing meal, regardless of whether a subject is deficient or not. We have found, however, that on low thiamine intakes, and when the thiamine is administered in divided doses, the rise in excretion does not occur, the output remaining relatively constant throughout the 24 hours at a level approximating the fasting excretion level. The 24-hour urine obtained under these conditions therefore yields the same information as the fasting excretion test and obviates the necessity of accurate timing of samples for collection; furthermore it provides larger quantities of material to work with.

Since Mickelsen, Castor and Keys ('47) have stressed the advantages of measuring the "pyramine" rather than the thiamine excretion, it may be in order to point out why we preferred the latter. Although these authors called attention to the greater individual variations in thiamine than in "pyramine" excretion, this was not true of the low range bordering on deficiency with which we were particularly concerned. An even more important consideration is the fact that the thiamine excretion curve, unlike the "pyramine" curve, shows a sharp break which makes a convenient, and we believe a significant, point of reference. Unlike "pyramine" excretion, which decreases in a continuous exponential curve as the intake is reduced, thiamine excretion decreases as a linear function of the intake until a certain critical point is reached which we have termed the "point of minimum excretion." Decreasing the intake beyond this point causes only a minimum further decrease in thiamine excretion. We have observed this phenomenon in all adults and infants we have studied. Evidence of it may also be found in the observations of Knott ('40) and of Mason and Williams ('42). Our inter-

pretation of this phenomenon, which is shared by Mason and Williams, is that the higher excretion rate above this critical point represents a surplus above minimum physiological needs. This hypothesis was tested experimentally by Najjar and Holt ('43)[2] in adult subjects who were carried to the point of thiamine deficiency symptoms. The point of minimum urinary excretion was attained when the intake was reduced to 0.6 mg per day; when the intake was gradually reduced beyond this point during a period of many months, frank deficiency symptoms were noted at an intake of 0.2 mg per day. Since undetected tissue alterations may well have occurred some time before the appearance of the frank symptoms, it seems probable that the beginning of deficiency is associated with an intake not far below that which gives rise to "minimum excretion." Further support for this point of view was obtained by Salcedo et al. ('48) in experiments on thiamine-depleted rats in which the concentration of thiamine in various tissues was correlated with the development of minimum urinary excretion. The course of depletion was associated with a progressive decrease in thiamine concentration in the liver, kidney and heart; the brain, on the other hand, maintained its concentration of thiamine until the point of minimum excretion was reached, after which the thiamine content fell abruptly.

Since the data referred to above indicated that the "point of minimum excretion" was apparently close to, if not identical with, the beginning of subclinical deficiency and that it could be attained with reasonable safety and without risk of clinical deficiency symptoms, we undertook to determine this point with accuracy in a group of infants as a measure of the minimum requirement for thiamine.

EXPERIMENTAL

Subjects

The subjects of this study were 7 healthy male infants. Five ranged between 7 and 10 months of age, weighing between

[2] See also Holt ('44).

TABLE 1

Composition of the salt mixture[1]

INGREDIENT	QUANTITY	INGREDIENT	QUANTITY
	%		%
$NaCl$	18.9	Fe citrate	2.21
CaH_2PO_4	25.0	$CuSO_4$	0.24
$MgSO_4$	6.8	$MnSO_4$	0.15
$KHCO_3$	44.4	KI	0.015
KCl	2.88	NaF	0.03

[1] Incorporated as 5 gm of mixture per 750 Cal. of diet.

5.9 and 7.7 kg at the start; the other two were one month and three months of age and weighed 4.1 and 4.7 kg respectively. They were placed on metabolism frames for a 5-day period each week during which 24-hour urine specimens were collected; this was followed by a two-day rest period.

Diet

In order to secure a basal diet essentially free of vitamins, to which vitamins could be added in accurately weighed quantities, a purified diet was employed consisting of vitamin-free casein, vitamin-free dextrimaltose,[3] hydrogenated cottonseed oil,[4] and a salt mixture (table 1); these constituents were mixed with water to provide a feeding, cereal-like in consistency. The diet was so composed that 50% of the calories were supplied by the carbohydrate, 35% by the fat and 15% by the protein. One hundred calories per kilogram of body weight were fed daily. All the vitamins were administered as supplements. Vitamins A and D were given in the form of oleum percomorphum, 15 drops daily. The water-soluble vitamins (table 2) were divided into 4 equal doses and administered midway between meals; this was done to avoid the spilling that occurs in the urine as a result of a single large dose.

[3] Specially prepared by Mead Johnson & Company through the courtesy of Dr. Warren M. Cox, Jr.

[4] Crisco. Contributed by Procter and Gamble.

Laboratory methods

Urine was collected in dark bottles containing sufficient glacial acetic acid to make a 2% solution. The thiamine content of each 24-hour specimen was determined fluorimetrically

TABLE 2

Composition of vitamin mixture (daily dose)

VITAMIN	QUANTITY	VITAMIN	QUANTITY
	mg		*mg*
Thiamine chloride	varied	Para-aminobenzoic acid	0.167
Riboflavin	0.167	Choline	0.833
Nicotinamide	4.16		
Pyridoxine	0.167	Inositol	0.167
Calcium pantothenate	0.167	Ascorbic acid	4.0–12.0 [1]

[1] The small dose (4.0 mg per day) was given to three infants in this study. It was calculated to provide an adequate requirement on the basis of an adult study (Najjar et al., '44) in which it had been shown that 25 mg per day would protect a 70 kg adult, the assumption being that the infant's requirement would be proportional to his weight. This intake proved adequate for two infants but inadequate for a third, who suffered from repeated attacks of otitis media and who developed clinical scurvy 6 weeks after his first illness and 5 months after he had been placed on this regimen. This precipitation of scurvy in the face of infection is in accord with the findings of Hamil et al. ('38), who found that 10 mg was adequate during health but failed to give full protection during periods of infection. After this incident the ascorbic acid intake was raised to 12 mg.

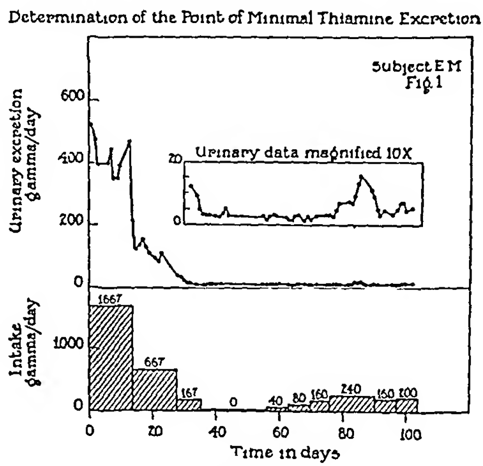

Figure 1

by the thiochrome procedure of Najjar and Ketron ('44). It should be emphasized that at these low levels of thiamine excretion the introduction of a correction factor for the presence of fluorescent derivatives of nicotinic acid, as is done in the Najjar-Ketron method, becomes a matter of great importance if accurate measurements of thiamine are to be obtained.

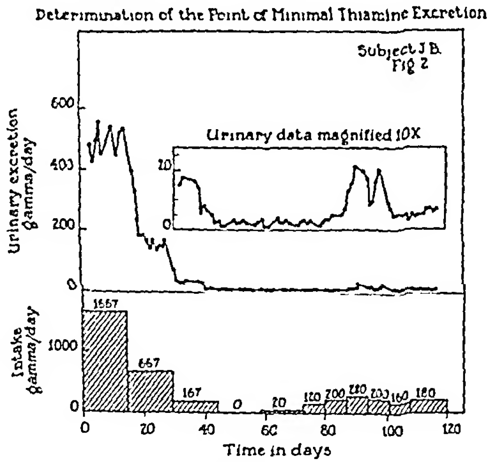

Figure 2

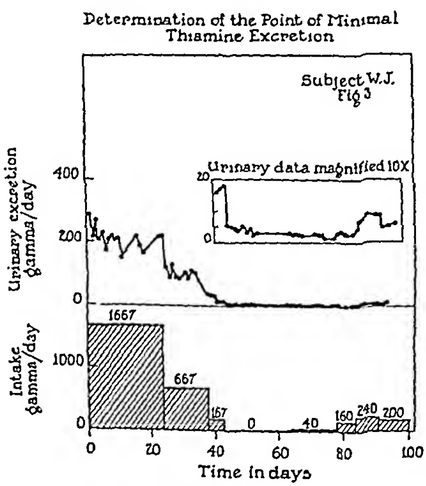

Figure 3

. . . experiment

. . . point of minimum excretion" of . . . possible, the following procedure was . . . on a high thiamine intake (1667 . . . thiamine was entirely omitted for a . . . permit the urinary excretion to be- . . . minimum level. The periods of high

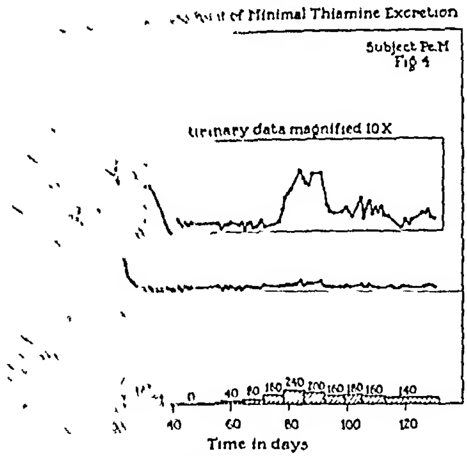

Figure 4

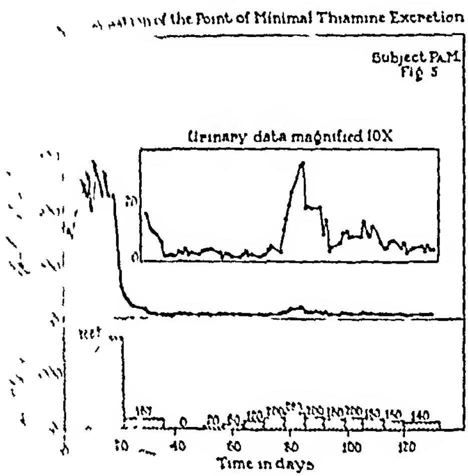

Figure 5

intake were omitted in the last two subjects; they were started immediately on a period of zero thiamine intake. Thiamine was then added cautiously in small amounts, increasing the intake at intervals of a week or two until excretion beyond the minimum occurred. The thiamine intake was then adjusted by subsequent small decreases and increases until an intake was obtained which would maintain excretion just above the minimum point.

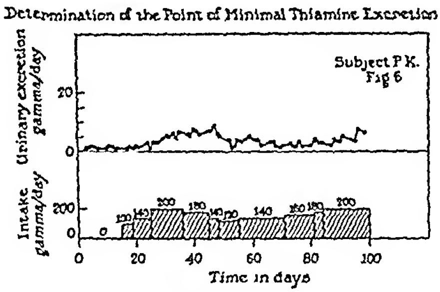

Figure 6

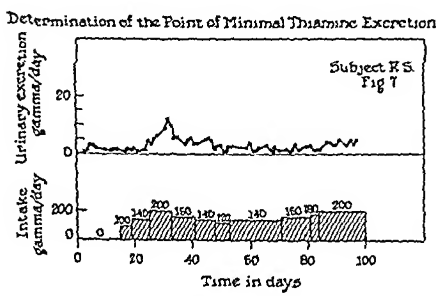

Figure 7

RESULTS

Protocols of the 7 subjects studied are shown graphically in figures 1–7. It is apparent that the minimum excretion in these infants corresponds to a 24-hour output of 1 to 5 μg per day. There appeared to be a slight variation among subjects; the smaller figures were more regularly encountered in the smaller subjects. A similar variation was found by Najjar

Plan of experiment

In order to ascertain the "point of minimum excretion" of thiamine as quickly as possible, the following procedure was followed. After two periods on a high thiamine intake (1667 and 667 μg per day) the thiamine was entirely omitted for a period of 14 to 21 days to permit the urinary excretion to become stabilized at the minimum level. The periods of high

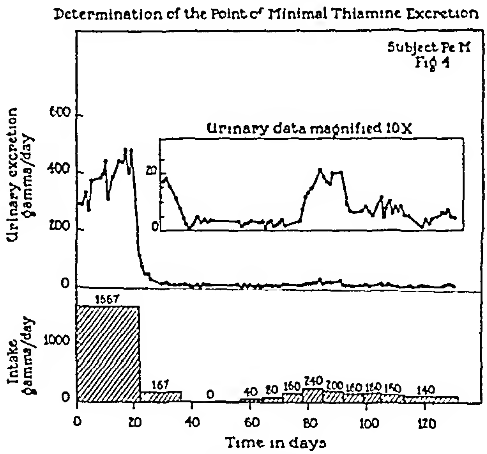

Figure 4

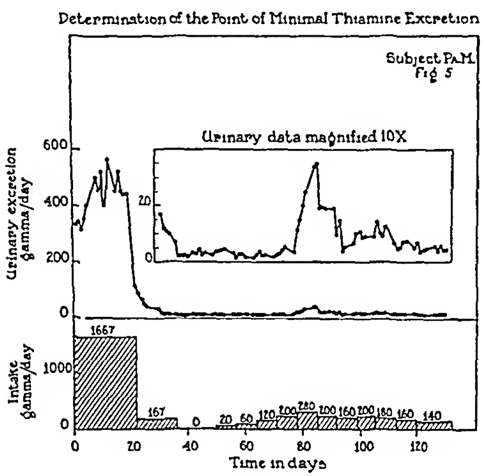

Figure 5

Mickelson et al. ('47), although, as we have mentioned, considerable variations were encountered in their study. The relation between thiamine excretion and intake in our infants appears to be far more constant at higher levels of intake than that observed by the Minnesota workers in their adult subjects.

Despite the fact that our subjects were maintained for 4 to 5 months on a low thiamine intake close to what we have

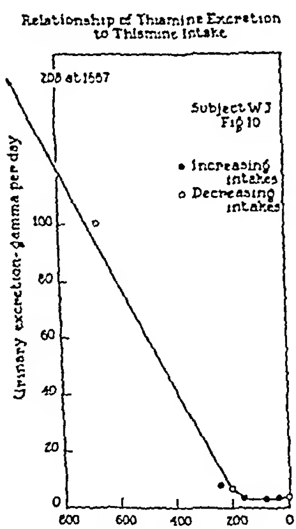

Figure 10

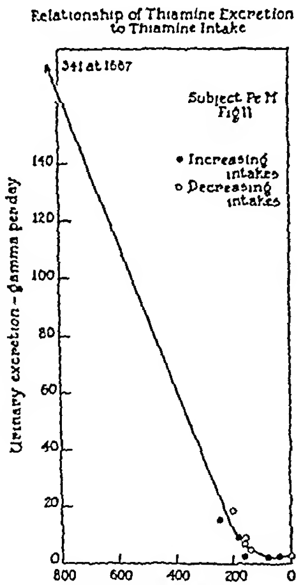

Figure 11

regarded as the minimum, no signs or symptoms of thiamine deficiency developed. As far as could be ascertained by daily physical examinations with special search for neurological abnormalities, careful observation of the infants' behavior, and laboratory data such as complete blood counts, urinalyses, x-rays of the long bones, and electrocardiograms, these infants continued to progress in an essentially normal fashion. However, after approximately 6 months on the purified diet,

and Holt ('43) in adults, in whom minimum excretions of between 36 to 60 μg per day (1.5 to 2.5 μg per fasting hour) were observed.

The intake which will produce an excretion just in excess of the minimum level proved to be singularly constant in these infants, being 0.14 mg in one subject, 0.16 in two subjects, and 0.20 in the remaining 4. Reduction of intake below these amounts invariably resulted in an output of less than

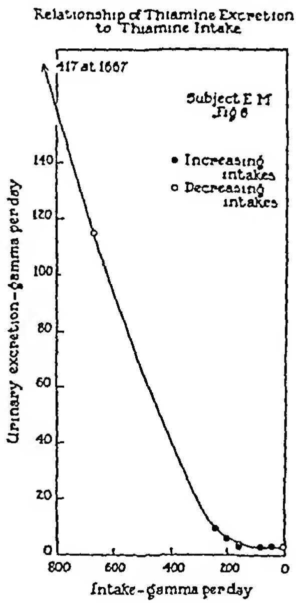

Figure 8

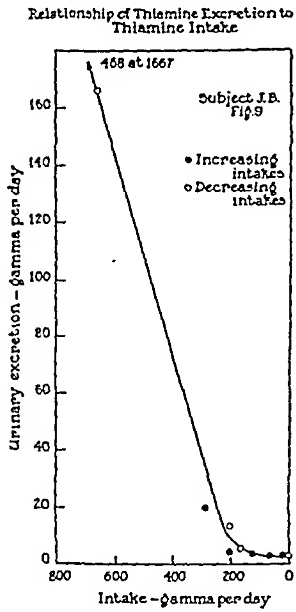

Figure 9

5 μg per day in the urine, whereas these quantities would maintain an output of between 5 and 10 μg per day. Any further increase produced a sharp rise in thiamine excretion.

The relation between intake and excretion is shown graphically in figures 8–12. The linear relationship between urinary excretion and intake is clearly seen with intakes above the critical point. Roughly one quarter of the added thiamine is excreted as such in the urine, a proportion slightly greater than that reported for adults in the extensive studies of

Mickelson et al. ('47), although, as we have mentioned, considerable variations were encountered in their study. The relation between thiamine excretion and intake in our infants appears to be far more constant at higher levels of intake than that observed by the Minnesota workers in their adult subjects.

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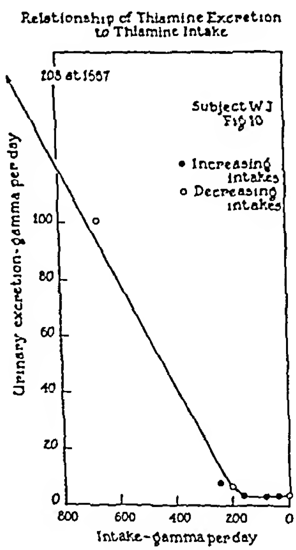
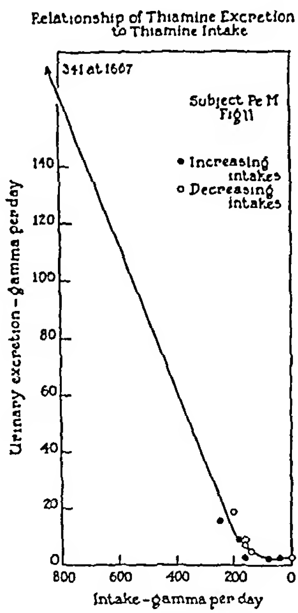


Figure 10



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we quite regularly encountered a diminution or cessation of gain in weight. This is not related to thiamine lack, since prolonged administration of large amounts of thiamine at this point does not alter the weight curve. Further evidence that thiamine deficiency was not concerned in this failure to gain weight was furnished by unpublished experiments in which similar behavior was noted when the same purified

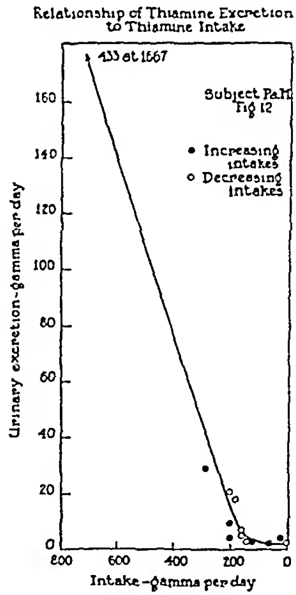

Figure 12

diet was fed without any thiamine restriction. This disturbance must be attributed to some other factor deficient in the synthetic diet; we have noted that it could be corrected by brewers' yeast in the majority of cases in which this was tried. In the two subjects (P. K. and R. S.) in whom it was possible to attain the endpoint rapidly, weight gain proceeded at a very satisfactory rate despite the restriction of the thiamine intake to from 0.14 to 0.20 mg daily for a considerable period of time.

COMMENT

It is of interest to compare the minimum thiamine requirement of 0.14 to 0.20 mg per day, as determined by this technique, with the intake which the average artificially-fed infant receives. Based on an average content of 35 to 40 µg of thiamine per 100 ml of cow's milk, an infant weighing 7 kg would receive at least 300 µg a day, apparently an ample margin of safety beyond a daily requirement of 200 µg. On the other hand, the destruction of thiamine by heat in the pasteurization and sterilization of milk formulas must be considered. According to Kon ('47), 10% of the thiamine may be destroyed by the former process and 30% or even more by more drastic sterilization procedures. The margin of safety with sterilized milk would thus appear to be very small indeed, and the possibility that autoclaving milk, which is becoming increasingly popular, may destroy even more thiamine is one that demands careful study.

Since breast milk contains roughly half as much thiamine as cow's milk, the margin of safety it provides would seem to be decidedly small. We hesitate to draw such a conclusion from present data, however, since there is reason for believing that some thiamine is formed from intestinal bacteria and it is possible that the flora of the breast-fed infant provides more of a supplement than that of the infant on a cow's milk diet. The data in the literature are inadequate to settle this point. A purified diet simulating more closely the composition of breast milk should throw light on this question. It should be emphasized that the figures in our study are applicable only to the conditions prevailing therein.

SUMMARY

The thiamine requirement of normal infants was determined by means of a urinary excretion procedure which involved determining the intake that would maintain urinary excretion at the upper limit of the zone of minimum excretion. The requirement varied between 0.14 mg and 0.20 mg per day in the 7 subjects studied.

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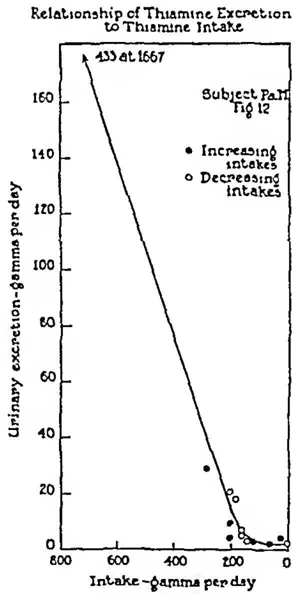

Figure 12

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VITAMIN STUDIES IN MIDDLE-AGED AND OLD INDIVIDUALS

II. CORRELATION BETWEEN VITAMIN A PLASMA CONTENT AND CERTAIN CLINICAL AND LABORATORY FINDINGS [1]

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FIVE FIGURES

(Received for publication August 26, 1948)

Although the literature dealing with the clinical aspects of hypovitaminosis A is quite extensive, few reports are available on the correlation between vitamin A plasma values and the objective symptoms presented by individuals. The present study was undertaken with the purpose of supplying such data.

The study included estimation of the frequency of occurrence of such signs as are generally accepted as being associated with a condition of insufficient intake of vitamin A, e.g., impaired dark adaptation, xerosis of the conjunctiva, Bitot's spots, blepharoconjunctivitis, dryness and scaliness of the skin, and follicular hyperkeratosis (toad skin). In addition to these clinical observations, studies were undertaken with the purpose of evaluating the degree of epithelial damage. The procedures employed included a determination of the daily excretion of epithelial cells in the urine and an examination of the percentage of keratinized epithelial cells in the urinary sediment. Furthermore, smears were made from the conjunctiva of both eyes and the percentage of cornified cells present was calculated from a differential count of the stained smears.

[1] Funds provided by Hoffmann-La Roche, Inc., Nutley, New Jersey.

The technique of plasma vitamin A estimation used and the values observed in a large group of individuals of different ages have been reported in a previous publication (Kirk and Chieffi, '48). The subjects included in the present investigation were chosen from the same group of inmates and patients in the St. Louis City Infirmary and Infirmary Hospital.

EXPERIMENTAL

Examinations for xerosis of the conjunctiva, localized conjunctival thickening, blepharoconjunctivitis, dryness and scaliness of the skin and toad skin were made on 106 subjects.

The examination for *xerosis* was performed by everting the lower eyelid and exposing the palpebral and bulbar conjunctiva to the air for two minutes. In no instance was dryness of the conjunctival mucous membrane noted after this period of exposure.

The percentage frequency of the occurrence of the other symptoms is listed in table 1, which also contains the calculated correlation significance both with regard to the plasma vitamin A concentration and to age. The various findings for each symptom will be discussed below.

Localized thickening of the conjunctiva

The conjunctival changes characteristic of vitamin A hypovitaminosis have, in the last few years, been the subject of much discussion. The conjunctival spots, as first described by Bitot in 1863, in patients suffering from poor dark vision were located just lateral to the cornea in the horizontal meridian of the eye, generally being in the shape of a triangle the base of which was slightly concave and about 5 mm in width, while the sides of the triangle were about 8 mm in length; the spots were usually covered by a white or yellow foamlike substance, presumably consisting of metaplastic epithelial cells. In an investigation by Kruse ('41), such Bitot "spots" were claimed to have been observed in a large percentage of the general population. The identification of

TABLE 1

Correlation of various clinical signs in 106 individuals with vitamin A concentration of plasma and with age

SYMPTOMS	Plasma vitamin A in μg%		"t" [1] test	Plasma vitamin A in μg%		"t" [1] test	Plasma vitamin A in μg%		"t" [1] test	Plasma vitamin A in μg%		"t" [1] test	Plasma vitamin A in μg%		"t" [1] test
	1–15	25–60		1–15	25–60		1–15	25–60		1–15	1–15		25–60	25–60	
	Age in years			Age in years			Age in years			Age in years			Age in years		
	16–99	16–99		16–59	16–59		60–99	60–99		16–59	60–99		16–59	60–99	
	Number of persons			Number of persons			Number of persons			Number of persons			Number of persons		
	61	45		18	21		43	24		18	43		21	24	
	%	%		%	%		%	%		%	%		%	%	
Localized conjunctival thickening	21	2	7.3	4	0	1.0	29	4	3.1	4	29	3.0	0	4	1.0
Blepharo-conjuncti-vitis	30	16	1.8	30	10	1.5	31	25	0.6	30	31	0.1	10	25	1.4
Dryness and/or scaliness of skin	64	42	2.3	30	16	1.0	81	67	1.3	30	81	3.9	16	67	4.2
Toad skin	44	9	5.0	35	0	3.2	50	16	2.2	35	50	1.1	0	16	2.2

[1] $\text{"t"} = \dfrac{\text{difference between averages}}{\text{standard error of difference}}$.

Kruse's conjunctival findings with Bitot's spots was, however, strongly contested by Berliner ('42), who suggested that the conjunctival infiltrations seen by Kruse were merely pingueculae, the changes being limited to the subconjunctival layer and not to the epithelium itself. According to Berliner, other raised spots may also be seen in very old individuals, consisting of yellow concretions of lipoid or hyaline material and white chalky deposits resulting from degeneration of cellular products. In a publication of the same year Kodicek and Yudkin ('42) expressed themselves as being in agreement with Berliner's criticism but suggested that the conjunctival lesion might be a forerunner of the Bitot spots and a sign of vitamin A deficiency. In view of the present uncertainty in the definition of various types of conjunctival spots, and in the absence of conjunctival biopsies, the authors have preferred to include the different observed lesions in one group, which in the table is designated as "localized conjunctival thickening." It appears likely that most of the pericorneal spots observed, although rather similar to the description given above, are not true Bitot's spots, as a large number were located medial to the cornea which is claimed rarely to be the case with the spots observed by Bitot.

Investigations of the correlation between plasma vitamin A values and the presence of conjunctival thickening as observed by slit lamp examination have been reported by Anderson and Milam ('45), who failed to demonstrate any certain relation between the conjunctival changes and the plasma vitamin A level but noted an increased incidence of conjunctival abnormalities with advancing age.

The results of our studies are reported in table 1. It will be seen from the table that the conjunctival thickening occurs with greater frequency in individuals with a low (1–15 µg%) than with a higher (25–60 µg%) plasma vitamin A content. Further analysis of the data indicates, however, that a combination of low plasma vitamin A content and old age appears to be important in the development of the conjunctival thickening.

The occurrence of *blepharoconjunctivitis* was also more frequent in individuals with a low than with a higher plasma vitamin A content, but the difference between the groups was not marked.

Dryness and/or scaliness of the skin

This symptom likewise occurred with greater frequency in patients with a low (1–15 µg%) than with a higher (25–60 µg%) plasma vitamin A level. A perusal of the columns in table 1 shows, however, that the effect of age is considerably greater than the influence of the vitamin plasma concentration, the frequency being much greater in the 60–99 year age group than in the 16–59 year old subjects. It should, however, be remembered in this connection that old individuals generally show less inclination for bathing than younger persons, a factor which may have influenced to some extent the above results, tending to aggravate the observed difference between the age groups.

Toad skin

The frequent occurrence of follicular hyperkeratosis in vitamin A deficiency was first described by Frazier and Hu ('31). The importance of this sign in the clinical picture of hypovitaminosis A has now been generally accepted, although it is believed that other factors may also be of etiological significance.

As will be seen in table 1, follicular hyperkeratosis was found with greater frequency in patients with a low vitamin A content than in individuals with larger stores of this vitamin, whereas its relation to age is less certain. In agreement with the observations of others, the toad skin was found most commonly on the extensor surfaces of the arms, forearms and thighs; in our observations the buttocks were also frequently affected. In young and middle-aged individuals the lesion consisted of dry, horny, round or oval sharply-defined protuding papules. In old subjects, however, the ap-

pearance of the eruption was somewhat different, the papules being smaller, only slightly elevated, and with a light yellowish or white center. It seems reasonable to assume that this difference was induced by the general atrophy of the skin so frequently present in old age.

Dark adaptation

Although the literature dealing with adaptation time in hypovitaminosis A is very extensive, observations on the correlation between the plasma vitamin A value, determined by a reliable technique, and the adaptation time have been reported only by Pett and Le Page ('40), who studied 26 male subjects between the ages of 20 and 30 years and found a definite relation between the two sets of values. The effect of age on dark adaptation has been studied by Phillips ('39), and by Robertson and Yudkin ('44). In both investigations a highly significant correlation was found, the correlation coefficient in Phillips' studies being + 0.57 to 0.81 and in Robertson and Yudkin's investigations + 0.56. A positive correlation coefficient is indicative of increased adaptation time with age. It was suggested by the latter authors that this impairment of dark adaptation with advancing years was caused by decreased size of the pupils in old individuals.

In the present study the dark adaptation was determined by the Feldman adaptometer.[2] Before being subjected to the test the patients were examined by opthalmoscopy and all individuals with lenticular opacities or other severe ocular lesions were excluded. Further, several patients were omitted because of senile psychosis or dementia. The screening of unfit individuals left only 77 for dark adaptation determinations.

The results, which are reported in figures 1 and 2, show no correlation between the vitamin A content of the plasma and the adaptation time (correlation coefficient —0.01) but a definite, though moderate, correlation between the age and

[2] American Optical Company.

the adaptation time (correlation ratio + 0.41). The latter finding is in agreement with the observations cited above. The reported figures do not permit conclusions concerning the cause of the impaired dark adaptation with age. It seems reasonable to assume that the size of the pupil, as suggested by Robertson and Yudkin, is of importance, but in the opinion of the present authors retinal arteriosclerosis with reduced

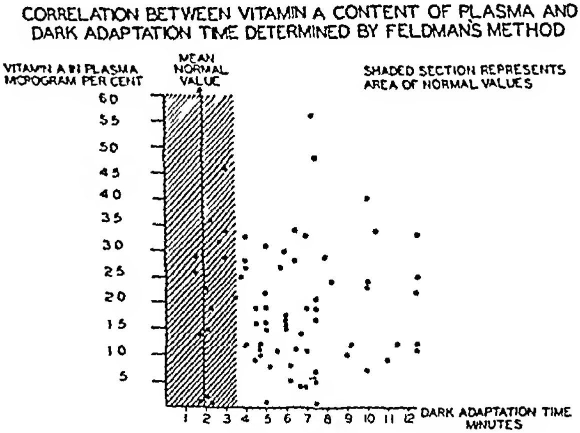

Figure 1

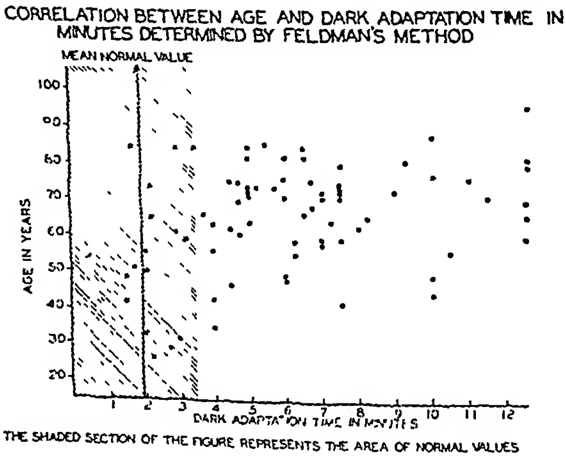

Figure 2

retinal blood supply, and cerebral arteriosclerosis with accompanying prolonged reaction time, cannot be ruled out as contributory factors.

Keratinized cells in conjunctival epithelium

According to the experience of Sweet and K'Ang ('35), the examination of conjunctival smears for keratinized epithelial cells constitutes a valuable method for the early diagnosis of hypovitaminosis A. No quantitative data in support of this contention are given, however, in their paper. A significant contribution to the subject was made in 1938 by Youmans, Corlette, Corlette and Frank, who examined conjunctival smears from 25 normal and 9 poorly fed individuals in order to establish criteria for the diagnosis of mild forms of deficiencies. Their results showed no significant difference between the proportion of keratinized cells and the nutritional state of the patient. The average per cent of cornified epithelial cells found was 59 (range 4–97%); the difference between the figures obtained from the two eyes of the same individual was frequently great, averaging 13%, with a range of from 4 to 34%.

In order to correlate, in the present study, the plasma vitamin A level and the percentage frequency of keratinized cells in the conjunctival epithelium, smears were made from both eyes of 83 individuals by rubbing cotton swabs over the conjunctivae of the everted lower lids. The smears were fixed in methyl alcohol and stained by Shorr's trichrome method ('41), a procedure which usually yielded very satisfactory preparations. A differential count was subsequently made of keratinized and non-keratinized cells in the smears from each eye of the same individual.

The results showed an average occurrence of keratinized cells from the right eye of 35% (range 0 to 100%, s.d. 26), and of 38% from the left eye (range 8 to 97%, s.d. 24). The difference between the two eyes of the same individual averaged 9.4% (range 0–37%, s.d. 7.9).

The mean value found for each of the individuals is plotted in figure 3 against the plasma vitamin A level. The results reveal a slight correlation between the plasma vitamin A values and the percentage of keratinized conjunctival cells, the correlation coefficient being —0.25. In view of this rather low degree of correlation and the frequent considerable difference between the values obtained from the two eyes of the same individual, this method of procedure hardly constitutes a useful approach to the clinical examination of patients with regard to plasma vitamin A concentration.

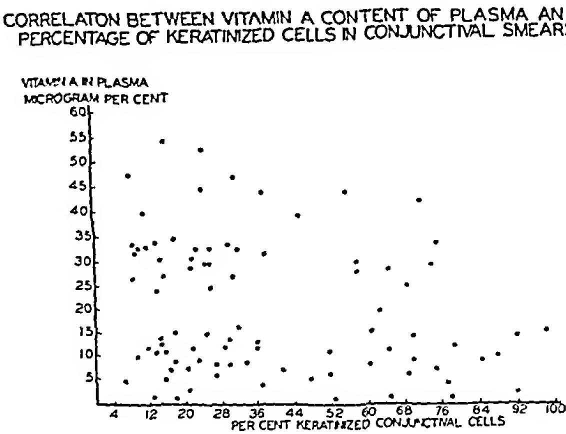

Figure 3

Epithelial cells in the urine

The data in the literature concerning the significance of an abnormal excretion of epithelial cells in the urine and an increased tendency to keratinization of those cells in subjects receiving a diet inadequate in vitamin A are very scarce, and quantitative measurements are entirely lacking. In a 1931 paper Spence comments on the great number of epithelial cells in the urine of children affected with xerophthalmia. In contrast with this, Sweet and K'Ang ('35) in examining fresh urine specimens from 13 children with clinical signs of hypovitaminosis A noted an abnormal number of squamous epithelial cells in only one case.

In the present study measures were undertaken to evaluate quantitatively the excretion of urinary epithelial cells and the frequency percentage of keratinization, and to correlate these findings with the plasma vitamin A concentration.

The sediment count was performed as described by Addis ('25). In consideration of the frequency of urination often encountered in old individuals the collection periods for the urinary samples were, however, often reduced to 2–4 hours, during which time the patients were under constant supervision. In the female patients the samples were collected by

CORRELATION BETWEEN VITAMIN A CONTENT OF PLASMA AND 24 HOURS URINARY EXCRETION OF EPITHELIAL CELLS

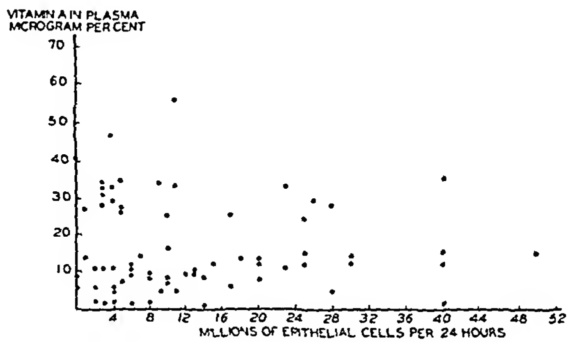

Figure 4

catheterization. The employment of a shorter period for the sample collection was not considered to introduce any error, as investigations by Naeraa ('36) have shown that essentially similar values are found by examination of 24-hour specimens and by calculation of the cellular output on the basis of fractional samples.

Eighty-four individuals were included in the investigation. In 13 cases the presence of urinary infection was noted. The plasma vitamin A levels for the latter individuals were 3, 5, 7, 9, 25, 27, 29, 30, 31, 40, 45, 46, and 48 µg% respectively (average, 25 µg%). As the mean vitamin A plasma concen-

tration for the 71 subjects without evidence of urinary infection was 15 μg%, these findings do not support the view that a low vitamin A content of the plasma is a factor of significance in the development of urinary infection in humans. The epithelial counts observed in the 71 cases are reported in figure 4. The data disclose no correlation between the plasma vitamin A value and the number of excreted epithelial cells, the correlation coefficient being —0.02.

Smears of the urinary sediment were fixed and stained by the same technique employed in the investigation of the con-

CORRELATION BETWEEN VITAMIN A CONTENT OF PLASMA AND PERCENTAGE OF KERATINIZED CELLS IN URINARY SEDIMENT

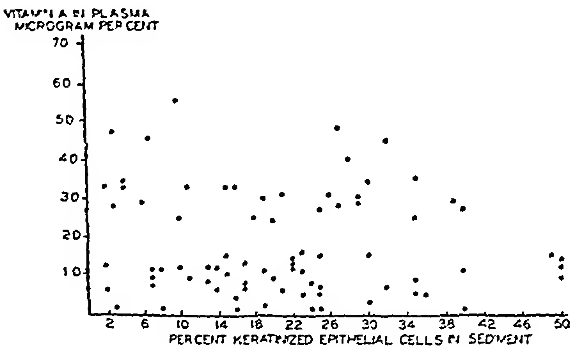

Figure 5

junctival samples. The keratinized cells were usually clearly distinguishable and could easily be subjected to differential counting. The results of the counts in the 71 samples which showed no evidence of infection are given in figure 5. The data show no certain correlation between the vitamin A value and the percentage of keratinized cells, the correlation coefficient being —0.20.

SUMMARY

A clinical study of 106 individuals with regard to signs of vitamin A deficiency revealed a considerably higher frequency of occurrence of hyperkeratosis of the skin follicles

and of localized conjunctival thickening in the subjects with a low vitamin A plasma level (1–15 µg%) than in those having a higher plasma vitamin concentration (25–60 µg%). The occurrence of dryness of the skin and of blepharoconjunctivitis was also more frequent in the subjects with hypovitaminemia A, but the difference between the groups was not marked.

No correlation was found between dark adaptation time and vitamin A plasma values but a significant, though moderate, correlation was observed between length of adaptation time and age.

The percentage of keratinized cells in conjunctival smears was slightly higher in indviduals with low vitamin A plasma values, but the percentage variation between different subjects and between the two eyes of the same subject was so great as to tend to invalidate the clinical usefulness of this examination.

No correlation was observed between the vitamin A plasma concentration and the number of epithelial cells excreted daily in the urine, nor between the plasma value and the percentage of keratinized cells in the urinary sediment.

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SELF SELECTION OF DIET

IX. THE APPETITE FOR THIAMINE [1]

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The concept of a learned or "educated" appetite was first advanced by Harris, Clay, Hargreaves and Ward ('33). Early studies were made by these workers on "vitamin B," while their later work was concerned with partially purified "vitamin B_1." It appears probable that their conclusions are applicable chiefly to thiamine. These investigators attempted to determine the limitations of a learned appetite, and discussed the possibility that such appetites may be of considerable importance in practical feeding. With the advantage of much more complete knowledge of the rat's nutritional requirements, this laboratory has attempted to investigate the nature of learned appetites for thiamine and other B vitamins and to evaluate their significance. This has resulted in the demonstration of such appetites (Scott and Quint, '46b), and in exploration of the role of learning in their inception (Scott and Verney, '47). The present paper describes certain experiments on the appetite of rats for thiamine-containing foods which attempt further to describe and explain the nature of a learned appetite.

EXPERIMENTAL

In the first series of 10 experiments the effect was studied of thiamine concentration, both in the previous diet and in

[1] This research was aided by grants from the Nutrition Foundation, Inc., and from the Buhl Foundation.

the thiamine-containing choices, on the appetite for thiamine-containing foods. The experimental method was that previously described in detail by Scott and Quint ('46a). In each experiment, 5 male and 5 female weanling rats in separate cages were offered during a three-week control period, a standard purified diet in each of two cups, while another similar group was given in the same way a thiamine-containing diet.[2] These 10 experiments differed among themselves only in the thiamine content of the latter diet. Other vitamins were given separately as pills.[3] Body weight and food consumed from each cup were determined daily and the cups then interchanged in a predetermined random manner. During a three-week experimental period both groups were given their choice, in separate cups, of the standard diet and the same thiamine-containing diet as was used in the control period. Body weight and food consumption from each cup were measured and the cups interchanged daily in the same manner as before.

To determine the effect of additional thiamine-free choices on the appetite for thiamine-containing food, an experiment similar to those above was devised in which, during the experimental period, three cups were filled with standard diet and only one with thiamine-containing diet (two μg per gram).

For reasons discussed below it was decided to study the eating behavior of rats when (1) a high level of thiamine in the diet was first associated with a flavor, or lack of it, and (2) when the flavor was changed with respect to the vitamin. The technique was exactly that previously described in detail (Scott and Verney, '47) except that the thiamine content of the diet was much higher. At the end of the control period one

[2] The standard diet consisted of 24% casein (Labco "vitamin-free"), 10% hydrogenated vegetable oil ("Primex"), and 4% salt mixture (Jones and Foster, '42). Thiamine-containing diets contained in addition 0.1 to 1000 μg/gm of thiamine hydrochloride.

[3] One pill was given each rat daily. It contained approximately 100 μg riboflavin, 90 μg pyridoxine hydrochloride, 150 μg calcium pantothenate, 10 mg choline chloride, 1 mg α-tocopherol, and 55 I.U. vitamin A and 11 I.U. vitamin D as 0.001 ml Natola, all in a dextrin-powdered sugar base.

group of 10 thiamine-deficient animals and another of 10 control animals (previously fed 100 μg per gram thiamine in their food) were subdivided and allowed their choice of (1) unflavored standard diet and flavored (10 p.p.m. oil of anise) thiamine-containing (100 μg per gram) diet, or (2) flavored standard diet and unflavored thiamine-containing diet. After 5 days the flavor was reversed with respect to the vitamin, so that animals previously offered choices (1) above now chose from (2), and those previously offered (2) received (1). After this second period of 5 days the original choices were offered for a third period of the same length. During a fourth period of 6 days the choices were the same as in the second period. The amounts of the vitamin-containing and vitamin-free diets consumed in each period were measured.

The final experiment was concerned with determining how deficient in thiamine an animal must be in order to have a definite appetite for thiamine-containing food. Ten male and 10 female weanling rats were divided into two groups so that each animal had a litter-mate pair of the same sex and weight in the other group. One group throughout a 6-week period was given its choice of a thiamine-containing diet (0.5 μg per gram) and the standard diet. The other group was given the same thiamine-containing diet in each of two cups. Body weight and food consumed from each cup were determined and the cups interchanged daily in a predetermined random manner.

RESULTS

The effects of thiamine concentration on appetite for thiamine-containing food are shown in table 1. The animals fed thiamine-containing diets (control groups) showed no evidence of deficiency in the control period if the thiamine concentration was 1 μg per gram or more, and no appetite for thiamine-containing food in the experimental period if the concentration was 2 μg per gram or more. At the highest level (0.1%) of thiamine, the diet containing the vitamin was avoided. The deficient groups all preferred the thiamine-

TABLE 1

Appetite for thiamine-containing food[1]

EXPT. NO.	THIAMINE[2] CONCENTRATION IN FOOD	CONTROL PERIOD (3 WEEKS) Weight change	Food eaten	Thiamine[2] intake: average	EXPERIMENTAL PERIOD (3 WEEKS) Weight[3] change	Food eaten	Thiamine[2] intake: average	Appetite[4] for thiamine
	$\mu g/gm$	gm	gm	$\mu g/day$	gm	gm	$\mu g/day$	%
				Control animals				
1	0.1	22.4 ± 2.3	88.5 ± 4.3	0.4	(10)	(14.3 days)	0.1	26.0 ± 9.2
2	0.2	28.5 ± 3.5	113.0 ± 6.3	1.1	−29.4 ± 4.7(5)	47.7 ± 5.0	0.3	31.5 ± 8.2
3	0.5	51.4 ± 3.2	129.2 ± 7.9	3.1	−22.3 ± 5.4	84.3 ± 9.4	1.6	30.4 ± 3.6
4	1	66.9 ± 4.8	154.9 ± 11.3	7.4	18.3 ± 6.6	151.9 ± 12.5	6.0	29.7 ± 8.8
5	2	72.9 ± 4.0	153.9 ± 8.2	14.7	60.1 ± 4.4	237.3 ± 8.7	14.3	8.3 ± 4.0
6	5	63.9 ± 4.6	155.2 ± 9.8	37.0	39.3 ± 6.3	199.1 ± 14.5	27.3	8.8 ± 6.9
7	10	65.5 ± 2.8	137.5 ± 7.3	65.5	35.8 ± 3.4	182.4 ± 10.7	42.1	−7.3 ± 7.9
8	20	63.0 ± 4.6	141.3 ± 7.0	134.5	58.9 ± 5.3	216.8 ± 10.9	87.6	−6.3 ± 7.2
9	100	61.3 ± 2.4	147.3 ± 6.5	701.4	64.6 ± 5.5	246.5 ± 9.5	753.7	10.7 ± 8.4
10	1000	70.6 ± 2.5	157.6 ± 4.3	7504.8	52.0 ± 4.5	223.4 ± 7.4	2724.0	−22.3 ± 4.3
				Experimental animals				
1	0	17.5 ± 3.3	82.2 ± 3.8	0	(9)	(12.0 days)	0.1	16.7 ± 9.7
2	0	11.3 ± 2.6	85.0 ± 6.3	0	(10)	(11.6 days)	0.2	7.1 ± 8.0
3	0	29.9 ± 1.7	99.6 ± 5.2	0	−15.1 ± 3.2	72.1 ± 4.9	1.3	20.9 ± 8.7
4	0	18.7 ± 2.0	93.1 ± 5.7	0	18.2 ± 6.9(4)	90.4 ± 9.0	3.1	35.6 ± 5.8
5	0	22.2 ± 3.0	91.0 ± 5.6	0	74.1 ± 5.4(3)	188.3 ± 14.2	11.1	19.7 ± 10.1
6	0	15.6 ± 2.2	94.0 ± 4.6	0	77.3 ± 4.5	189.2 ± 7.6	36.0	29.8 ± 5.2
7	0	20.4 ± 1.6	84.8 ± 3.2	0	56.9 ± 2.9	177.0 ± 8.4	65.8	23.8 ± 11.8
8	0	14.4 ± 3.4	86.8 ± 3.5	0	64.2 ± 4.8	159.9 ± 10.7	98.4	23.0 ± 5.7
9	0	20.4 ± 2.4	95.2 ± 5.0	0	81.1 ± 3.5	211.8 ± 8.0	776.7	29.8 ± 5.8
10	0	17.5 ± 2.2	87.5 ± 3.5	0	72.8 ± 3.2	192.4 ± 6.1	7678.8	41.1 ± 7.5

[1] All data are in terms of the mean and the standard error of the mean.

[2] Actually, thiamine hydrochloride.

[3] Figures in parentheses represent number of animals that died in the experimental period. These animals were excluded from the weight change and food intake data. Where the majority of animals died the figure given is the average length of life of the group in the experimental period.

[4] Average difference (in per cent of total food intake) between the intake of vitamin-containing food in the experimental period and the food intake from the corresponding cup in the control period. A significant positive value indicates a preference.

TABLE 2

Preference for thiamine-containing food as one choice out of 4

	CONTROL PERIOD		EXPERIMENTAL PERIOD					
					Preferences[1] for			
	Weight change	Food eaten	Weight change	Food eaten	Thiamine-containing choice	Other choices		
	gm	*gm*	*gm*	*gm*	%	%		
Control animals	78.0 ± 3.4	158.4 ± 5 6	66.1 ± 5.3	254.6 ± 10.4	43.4 ± 3.8	— 17 8 ± 7.7	— 15.8 ± 2.2	— 10.7 ± 1.9
Deficient animals	21 6 ± 2.5	93.2 ± 4.4	68.1 ± 6.0	171.1 ± 10.9	54.8 ± 7.5	— 23.0 ± 2.7	— 20.5 ± 3.4	— 12.4 ± 2.9

[1] Average difference (in per cent of total food) between the food consumed from a given cup in the control period and that consumed from the same cup in the experimental period. A positive value indicates a preference.

containing diet although, at very low thiamine concentrations, the condition of the rats was so poor that it may have made the results uncertain.

The results of the experiment in which the thiamine-containing food was one choice among three are presented in table 2. Both the control and deficient groups uniformly selected the thiamine-containing choice.

In the previously reported flavor study of thiamine (Scott and Verney, '47) it was found that the normal animals which

TABLE 3

Flavor change and preference for thiamine

THIAMINE LEVEL IN DIET	PART OF EXPERIMENTAL PERIOD			
	First	Second	Third	Fourth
p.p.m.				
	Difference in body weight change [2] (gm per day)			
5 [1]	-0.30 ± 0.56	-1.16 ± 0.73	0.46 ± 0.54	1.25 ± 0.46
100	0.86 ± 0.54	0.16 ± 0.42	-0.02 ± 0.56	-0.18 ± 0.45
	Difference in preference for thiamine [3] (per cent)			
5 [1]	57.2 ± 8.2	-11.0 ± 14.7	30.4 ± 12.0	-1.1 ± 13.6
100	47.0 ± 10.7	-15.4 ± 20.4	8.8 ± 12.5	-11.8 ± 12.3

[1] Data from Scott and Verney ('47).

[2] Average weight change of deficient animals minus that of control group.

[3] Average per cent thiamine-containing food selected by deficient animals minus per cent selected by control animals. A significant positive value indicates a greater preference by the deficient animals.

had received thiamine in the control period showed little or no preference in the experimental period. Accordingly it appeared proper to use them as a control group to which the deficient animals could be compared. This was done, as is shown in table 3, where the data from experiments using both a high level (100 μg per gram) and low level (5 μg per gram) of thiamine as a choice are summarized. In both experiments there was a pronounced preference by deficient animals for the thiamine-containing diet in the first part of the experimental period, and a slight avoidance of it in the second part

after the flavor was switched with respect to the vitamin. The results of the two experiments differed in the third part, with the deficient animals on the low-thiamine choice showing a distinct preference while those at the higher level showed only a slight preference. In the fourth part both deficient groups avoided the thiamine-containing diet to a slight extent. The differences in weight gains show a more rapid recovery of the animals selecting a high concentration of thiamine.

TABLE 4

Comparison of animals with and without choice of thiamine-containing food

WEEK	DIFFERENCE BETWEEN PAIRS			
	Wt. change [1]	Food eaten [1]	Thiamine intake [1]	Preference [2]
	gm/wk.	*gm/wk.*	*μg/day*	%
First	2.7 ± 1.2	1.6 ± 1.5	1.3 ± 0.3	9.9 ± 11.4
Second	6.9 ± 2.4	10.0 ± 2.7	2.3 ± 0.4	—3.6 ± 9.7
Third	11.8 ± 4.3	18.3 ± 6.8	1.9 ± 0.6	28.4 ± 11.5
Fourth	10.9 ± 4.7	25.3 ± 8.3	2.1 ± 0.7	35.5 ± 10.3
Fifth	8.8 ± 5.2	21.7 ± 10.6	1.7 ± 0.8	25.7 ± 10.5
Sixth	6.0 ± 3.7	15.6 ± 9.4	1.3 ± 0.7	29.8 ± 8.1

[1] Average of the figures for the animals fed only a thiamine-containing diet minus the figures of the corresponding pair mates given a choice. A positive value indicates greater weight gain, food consumption, or thiamine intake for the animals without choice.

[2] Average per cent of total food that was thiamine-containing selected by animals on choice minus the per cent of total food eaten from a corresponding cup by respective pair mates not given a choice. A significant positive value indicates a preference for thiamine-containing food by the animals given a choice.

In the final experiment weekly differences between pairs of animals, one of which had a choice of thiamine-containing and thiamine-free diets and the other only a thiamine-containing diet, were compared (see table 4). It will be noted that the animals given a choice grew significantly less as early as the first week and that they ate less food in the second week, but that not until the third week was a preference shown by them for the thiamine-containing food.

given a choice, they were in so much poorer condition than the controls that they could not catch up with their pair mates. The effect of their choice under these circumstances did not approach that which would result from the prescription of a good diet in promoting adequate nutrition.

In an evaluation of the possible importance in practical nutrition of the appetite for thiamine the following points should be considered:

(1) The appetite for thiamine is shown only by animals in a state of thiamine deficiency. There is no evidence of an "instinctive" appetite for thiamine. Thiamine appetite can lead to remission from thiamine deficiency but does not prevent its onset.

(2) The appetite for thiamine is not universal in all rats. Of 80 animals in these experiments who were previously fed thiamine-free diets and then offered a choice of a thiamine-free diet and one containing 1 p.p.m. or more of thiamine, two animals ate less than 10% of their total food from the thiamine-containing choice and 7 ate less than 50% of their food from that choice.

(3) A simple uncomplicated choice of the type obtained in these experimental studies will not ordinarily occur in actual feeding. Natural foods different in thiamine content will ordinarily be of different origins and will vary widely in flavor and in physical and chemical properties. Several other animals are much more selective in choice of food than are rats, and are markedly influenced by flavor. Food choice in man in particular is influenced by a large number of social, cultural and economic factors which, with the exception of education based on scientific nutrition, appear to bear little if any relation to nutritional needs. Thus an appetite for thiamine under most actual feeding conditions can scarcely be anticipated on the basis of our studies.

It appears, then, that a learned appetite for thiamine under certain conditions can lead to better nutrition than would wholly random eating, but that it cannot result in better nutrition than when the diet is prescribed on the basis of known

nutritional needs. In fact, under critical conditions such as are found in the final experiment above, self selection of diet for thiamine would ordinarily be inferior to obligatory feeding of thiamine-containing foods in promoting nutritional well-being.

SUMMARY

Rats suffering from thiamine deficiency show a preference for diets containing as little as 0.1 and as much as 1000 p.p.m. of thiamine. The appetite for thiamine-containing foods was not universal in thiamine-deficient rats and a small minority always made a poor choice. Rats fed a diet with a critically low concentration of thiamine were in better condition than rats offered a choice between this diet and a thiamine-free diet. Appetite for thiamine-containing foods was not established until the animals were distinctly deficient as judged by weight gain and food intake. It was concluded that while the learned appetite for thiamine can lead to better nutrition than would wholly random eating, it can at best only approach, and under critical conditions fails to approach, the benefit obtained by obligatory eating of a diet prescribed on the basis of known requirements.

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PHLORHIZIN DIABETES IN VITAMIN DEFICIENCIES[1]

CONVERSION OF PROTEIN TO GLUCOSE, AND SERUM PHOSPHATASE, DURING DEFICIENCIES OF THIAMINE, RIBOFLAVIN, AND PYRIDOXINE

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INTRODUCTION

Conversion of amino acids to glucose is a rather intricate phase of protein metabolism. Deamination, which is the first step, may be effected by way of transaminase and L-glutamic dehydrogenase. The latter enzyme requires coenzyme I, containing nicotinic acid. Transaminase activity in tissues is diminished during pyridoxine deficiency (Schlenk and Snell, '45), and the relationship appears to be a direct one (Ames, Sarma and Elvehjem, '47). McHenry and Gavin ('41), discussing the formation of fat from protein, suggested that pyridoxine may be required for preliminary conversion of protein to carbohydrate. Riboflavin deficiency might be expected to interfere with oxidation of glucose formed from protein, but no evidence can be cited in this connection.

These considerations, together with an interest in the effects of vitamin deficiencies in experimental pancreatic diabetes (Gaebler and Ciszewski, '45; Gaebler and Mathies,

[1] Data in this paper are taken from Part I of a thesis submitted by one of the authors (P.D.B.) to the Graduate Council of Wayne University in partial fulfillment of requirements for the degree of Doctor of Philosophy in Chemistry.

'46), led us to study the effects of thiamine, riboflavin, and pyridoxine deficiencies on the D:N ratio in phlorhizin diabetes. Nicotinic acid deficiency could not be included, since we used a diet containing over 72% of vitamin-free casein, which would provide for maximum synthesis of nicotinic acid from tryptophane. Since formation of glucose from protein takes place in the liver, several liver function tests were included in our studies. As a result, we obtained unexpected data on changes in the alkaline phosphatase of serum during phlorhizin diabetes.

EXPERIMENTAL

Adult female dogs were given a diet having the following percentage composition: Vitamin-free casein,[2] 72.2; corn oil, 19.8; Phillips-Hart ('35) salt mixture, 4; calcium phosphate, 4. Each animal received daily three drops of haliver oil, 500 mg of choline chloride, and the following amounts, in μg per kilo, of other vitamins: thiamine HCl, 50; riboflavin, 100; nicotinic acid, 500; pyridoxine HCl, 40; calcium pantothenate, 200. After a preliminary period of caloric adjustment, one vitamin was omitted. When characteristic signs of deficiency appeared, 1 gm of phlorhizin, finely suspended in olive oil, was injected morning and evening. In 5 experiments the deficiency was terminated after the third or fourth day of phlorhizin by administering large amounts of the missing vitamin. Phlorhizination and determination of D:N ratios were continued during the dramatic recovery which followed.

Urines were collected under toluene in 24-hour periods terminated by catheter. Nitrogen was determined by Kjeldahl digestion of samples containing about 5 mg, followed by distillation into 0.02 N acid and titration with 0.02 N alkali. Glucose was determined according to the method of Shaffer and Somogyi ('33) after diluting the urines 1:100. True sugar in zinc hydroxide filtrates of blood (Somogyi, '30) was determined with the same reagent. Hemoglobin values, of special interest in pyridoxine and riboflavin deficiencies, were deter-

[2] Labco.

mined with a Beckman spectrophotometer calibrated on the basis of oxygen capacity. Bromsulphalein in serum was determined in the manner described by one of the authors (Gaebler, '45), and alkaline phosphatase by the method of Bodansky ('33). The method of Van Allen ('25) was used for cell volume and that of Whitehorn ('21) for serum chlorides.

RESULTS

Dog 38 took the pyridoxine-free diet greedily for 18 days, but refused increasing amounts of it after 23 days. In spite of this, no anemia developed. On the 34th day the animal became completely incoordinated during play, and later had violent convulsions. The true sugar concentration in its blood just before this episode was 71 mg%. Normal appetite returned within 24 hours after 10.4 mg of pyridoxine were injected, and all untoward symptoms disappeared. Depletion was started a second time after the dog had, for 11 days, received the complete vitamin supplement already described. During 18 days of depletion the weight declined from 14.72 to 14.38 kg, increasing amounts of food were refused, and nitrogen balances became steadily more negative. All food was refused during phlorhizination, which was begun at the end of this period. As shown in table 1, the D:N ratios were quite low on the third and 4th days of phlorhizin administration but rose to 3.42 and 3.65, or an average of 3.54, when pyridoxine was given. Since the dog was consuming its own tissues, it may be pointed out that this value is in good agreement with the D:N of 3.49 reported by Janney and Csonka ('15) for dogs maintained on a diet consisting of dog muscle proteins.

Although dog 40 took the pyridoxine-free diet constantly and in adequate amount, its weight decreased steadily. Hemoglobin values fell uniformly, from 15.2 gm% on the 15th to 11.0 on the 30th day of depletion. On and after the 22nd day, 1 gm of desoxypyridoxine[3] was fed daily. Phlorhizination was begun on the 28th day. The D:N ratios are shown in

[3] We are indebted to Merck and Company for a liberal supply of this antivitamin, which is 2,4-dimethyl-3-hydroxy-5-hydroxymethyl pyridine.

table 1. During the first three phlorhizin days the animal refused about half of its food. When the antivitamin was stopped and pyridoxine was given, the dog resumed eating all of its food and the ratios rose. That the ratios on the second and third days of phlorhizin, during deficiency, were somewhat low is also indicated by reference to the control experiment shown in the last column of table 1. When this experiment was begun, dog 40 had, for 25 days, received a normal amount of pyridoxine.

TABLE 1

D:N ratios in phlorhizinized dogs during and after pyridoxine deficiency

DAY OF PHLORHIZIN INJECTION	DOG 38 DURING AND AFTER PYRIDOXINE DEFICIENCY	DOG 40 DURING AND AFTER PYRIDOXINE DEFICIENCY	DOG 40 LONG AFTER PYRIDOXINE DEFICIENCY
1	3.49	2.67	2.61
2	3.04	2.90	3.44
3	2.70	3.01	3.28
4	2.79	3.21 [2]	3.18
5	3.42 [1]	3.26	2.96
6	3.65	3.24	3.18

[1] Forty milligrams of pyridoxine were injected subcutaneously on the 5th and 20 mg on the 6th phlorhizin day.

[2] Feeding of desoxypyridoxine was discontinued. Twenty milligrams of pyridoxine were injected and 60 mg given by mouth. On and after the 5th phlorhizin day the vitamin supplement was complete.

The significant data of a riboflavin deficiency experiment on dog 40 are condensed in table 2. During the first 37 days, while food consumption was complete, the weight actually rose; thereafter it declined irregularly. Evidence of itching, and seborrhea, appeared as early as the 20th day; anorexia, on the 38th day. A 5-day phlorhizin period was begun on the 39th day and a second 6-day period on the 57th day of deficiency. At the latter time hemoglobin had fallen to 10.9 gm%. After the second phlorhizin period all deficiency symptoms became severe. Food was refused completely after the 68th day, the hemoglobin was 9.1 gm%, itching and seborrhea were

RIBOFLAVIN

days	kg	% of normal	gm	gm		gm%	%	units	%
8	9.98	100				14.90	42.5		
11	10.02	100				14.70	41.3		
14	9.98	100				14.95	43.5		
23	10.13	100				14.50	42.0	5.6	
29	10.24	100				14.95	43.1	5.5	
37	10.46	100				13.80	40.0		
38	10.02	50							
39 [1]	10.13	100	22.13	45.51	2.06				
40	9.79	50	21.60	53.80	2.49				
41	9.68	50	17.50	57.23	3.27			12.5	
42	9.57	100	22.99	66.88	2.91				5.2
43	9.68	100	22.00	64.80	2.94			11.1	
49	9.90	100				12.4	37.6	8.7	
56	9.83	83				10.9	32.5	7.7	3.2
57 [2]	9.57	50	13.05	43.00	3.30				
58	9.57	50	14.50	46.40	3.20				
59	9.23	50	16.60	52.00	3.13				
60	9.12	50	16.78	55.60	3.30				
61	9.23	100	21.35	63.60	2.98				
62	9.12	100	22.55	65.70	2.91				
63	9.12	100						5.4	
66	9.12	91				9.10	29.0	5.6	
68		0							
75 [3]	9.19	100				9.10	29.0		
87	9.76	100				11.75	35.8		
93	9.76	100				12.20	36.0		

[1] One gram of phlorhizin injected subcutaneously at 9 A.M. and again at 5 P.M., 39th to 43rd day.

[2] Phlorhizination repeated in the above manner, 57th to 62nd day.

[3] One hundred milligrams of riboflavin given by mouth daily on the 75th, 76th, and 77th days; complete supplement given thereafter.

marked, loss of hair around the eyes produced the "spectacle eye" appearance, and there was evidence of photophobia. All changes reversed after feeding of riboflavin was resumed on the 75th day. The D:N for the third, 4th, and 5th phlorhizin days averaged 3.04 in the first experiment and 3.14 in the second. The ratio reported by Janney ('15) for dogs on casein diets is 3.08. Bromsulphalein retention of the 5 mg per kilo dose at 30 minutes showed no evidence of liver damage. The upper limit of 15% retention in normal dogs reported by Drill and Ivy ('44) corresponds to 6% in our terms, since they used the 5 mg per kilo dose of bromsulphalein but preferred the standards corresponding to 2 mg per kilo. The rise in alkaline phosphatase shown in table 2 is clearly associated with phlorhizination, not with riboflavin deficiency, and will be discussed later.

Results of a riboflavin deficiency experiment in another animal, dog 43, are shown in table 3. Since loss of weight and nitrogen began almost immediately after riboflavin was withdrawn, the food intake was increased 25% after the first 12 days. Although the weight was stable for the next 18 days, nitrogen loss continued after a single three-day period. Anorexia and vomiting started on the 42nd day, but the animal resumed eating and phlorhizin was started on the 48th day. In the previous experiment we compared D:N ratios of two phlorhizin periods representing moderate and severe riboflavin deficiency; in the present experiment, the deficiency was terminated during the phlorhizin period. The ratios for the third and 4th phlorhizin days, during deficiency, averaged 3.26; those for the 5th and 6th, after riboflavin therapy was begun, averaged 3.19. The rise in serum phosphatase confirms the finding in the previous experiment.

Observations made during thiamine deficiency are recorded in table 4. Dog 43 lost weight steadily, vomited on the 23rd and 24th days of depletion, and was unable to retain either food or water on the 28th day. Dramatic improvement occurred on the 29th day when thiamine was injected. Food was consumed and retained, and the animal was active during

TABLE 3

Nitrogen equilibrium, glucogenesis, and other functions during riboflavin deficiency

TIME AFTER WITHDRAWAL OF RIBOFLAVIN	WEIGHT	FOOD CONSUMPTION	NITROGEN BALANCE PER DAY	URINE NITROGEN PER DAY	URINE SUGAR PER DAY	D:N RATIO	HEMOGLOBIN	CELL VOLUME	SERUM PHOSPHATASE (BODANSKY, '33)
days	*kg*	*% of normal*	*gm*	*gm*	*gm*		*gm%*	*%*	*units*
1–3	13.01	100	—0.18						
4–6	12.89	100	—1.02						
7–9	12.75	100	—0.79						
10–12	12.71	100	—0.85						
13–15	12.55	125 [1]	0.70						
16–18	12.71	125	—0.90						
19–21	12.75	125	—0.13						
22–24	12.71	125	—1.24						
26	12.67	125					15.7	43.0	
30	12.59	125					15.3	44.0	
46	12.03	125					16.8	47.9	9.0
47	12.14	125					13.4	39.0	6.5
48 [2]	11.91	63		13.70	34.60	2.52			
49	11.80	0		9.05	30.25	3.34			
50	11.57	0		8.38	27.95	3.34			
51	11.12	0		8.55	27.15	3.18	14.1	40.5	11.0
52 [3]	10.88	63		10.12	32.48	3.21			
53	10.88	125		14.60	46.24	3.17			
54	11.12	125		15.84	47.92	3.02			
55	11.08	125					13.3	40.0	7.4
65	11.00	125					13.0	37.0	4.3

[1] Food intake increased in an attempt to reverse negative nitrogen balances.
[2] One gram of phlorhizin injected twice daily 48th to 54th day, inclusive.
[3] One hundred milligrams of riboflavin given orally, followed by 50 mg next day, and a completely supplemented diet thereafter.

the morning exercise period. The D:N ratios on the third and 4th phlorhizin days averaged 3.38; those on the 5th and 6th, during thiamine therapy, averaged 3.42. Serum phosphatase increased markedly during phlorhizin administration.

TABLE 4

Gluconeogenesis from protein during thiamine deficiency

TIME AFTER WITHDRAWAL OF THIAMINE	WEIGHT	FOOD CONSUMPTION	URINE NITROGEN PER DAY	URINE SUGAR PER DAY	D:N RATIO	SERUM PHOSPHATASE (BODANSKY, '33)
days	*kg*	*% of normal*	*gm*	*gm*		*units*
			Dog 43			
3	12.85	100				
6	12.70	100				
9	12.66	100				
12	12.47	100				
13	12.43	100				1.3
22	12.32	100				0.7
25 [1]	12.14	100	12.62	46.95	3.72	
26	11.90	50	14.76	46.88	3.18	
27	11.79	0	11.67	41.48	3.56	7.4
28	11.11	0	14.22	45.45	3.19	
29 [2]	10.88	50	12.75	42.75	3.35	
30	10.88	50	9.80	34.20	3.49	
31	10.77	100	15.00	53.80	3.59	8.3
			Dog 44			
3	14.88	100				2.3
12	14.76	100				3.0
21	14.68	100				1.9
22 [3]	14.61	100	18.50	53.93	2.92	
23	14.27	0	14.50	49.43	3.40	
24	13.82	0	14.35	47.78	3.32	
25	13.48	0	13.20	42.30	3.20	5.0
26 [4]	13.24	50	12.60	45.80	3.64	
27	13.35	100	15.10	45.70	3.04	

[1] One gram phlorhizin injected twice daily for 7 days.

[2] One hundred milligrams of thiamine-HCl injected at 9 A.M. and again at 5 P.M. On the following day the complete vitamin supplement plus 50 mg of thiamine-HCl was added to the food.

[3] One gram of phlorhizin injected twice daily for 6 days.

[4] One hundred milligrams of thiamine-HCl injected at 8 A.M. and again at 5 P.M. The complete vitamin supplement was added to the diet.

Dog 44 likewise showed loss of weight, and anorexia appeared on the 22nd day of depletion. During the second, third, and 4th days of phlorhizin administration all food was refused, but the appetite returned promptly after thiamine was given. The D:N ratios on the third and 4th days of phlorhizin, during thiamine deficiency, averaged 3.26; those on the 5th and 6th days, during thiamine therapy, averaged 3.34. Serum phosphatase again showed an increase during administration of phlorhizin.

DISCUSSION

It seems worthy of comment that thiamine deficiency appeared in our dogs in the usual three weeks although the diet was carbohydrate-free and contained over 72% of protein. On a similar diet, Dann ('45) maintained rats in excellent condition for more than a year, although stunting occurred.

Since the central nervous system disturbances noted in one of our dogs are not frequently seen during pyridoxine deficiency in this species, it should be stressed that the anemia characteristic of this deficiency did not develop in this animal, and that there was no hypoglycemia. Glucose and oxygen were therefore available, whether or not they were utilized normally. It is also of interest that Morgan, Groody and Axelrod ('46) observed pyridoxine deficiency in young dogs after 169 to 190 days of depletion when the diet contained 18.0% of casein. On a diet containing 45.8% of casein, deficiency appeared in 79 to 123 days. In our experiments on adult dogs receiving 72.2% of casein the time was still shorter. Morgan, Groody and Axelrod reported elevation of blood sugar and chlorides during pyridoxine deficiency. Elevation of blood sugar did not occur in our experiments, but our diet was free of carbohydrate. Serum chloride values, however, were all between 640 and 694 mg% as NaCl.

The lowest D:N ratios, observed in dog 38 during pyridoxine deficiency, would correspond to 44% of glucose from protein instead of 58%. Formation of glucose was therefore not seriously impaired even in this animal. However, the

consistent results in two animals with proved pyridoxine deficiency, together with theoretical considerations mentioned in the introduction to this paper, make the results as they relate to pyridoxine deficiency at least suggestive, particularly in the absence of similar findings in other deficiencies. The elevation of D:N ratios as riboflavin deficiency progressed in dog 40, and the diminution after termination of this deficiency in dog 43 (see text, page 98) are too small to be taken as evidence that oxidation of glucose formed from protein was impaired.

The observation that serum alkaline phosphatase activity increased during administration of phlorhizin is of considerable interest, and reference should be made to a number of pertinent studies. Marsh and Drabkin ('47) observed an increase in both acid and alkaline phosphatase activities of the kidney during alimentary hyperglycemia in rats. *In vivo* and *in vitro* inhibition of both acid and alkaline kidney phosphatase activities by phlorhizin was reported. Increases in both acid and alkaline liver phosphatase activities (Drabkin and Marsh, '47) and increases in serum alkaline phosphatase activity (Cantor, Tuba and Capsey, '47) have been reported in alloxan-diabetic rats. Treatment of these rats with insulin is reported to have restored the phosphatase activities to normal. The elevation of serum alkaline phosphatase which we observed in phlorhizinized dogs may be an adaptive response to inhibition of phosphatase activity by phlorhizin. It may also be related to the disturbance of carbohydrate metabolism in phlorhizin diabetes.

SUMMARY

1. In dogs phlorhizinized during thiamine or riboflavin deficiencies of sufficient duration to produce the characteristic signs, formation of glucose from protein is not impaired. There is also no evidence that these deficiencies restrict utilization of glucose in the phlorhizinized animal.

2. In pyridoxine deficiency, the D:N ratios were quite low in one animal and somewhat low in another, and increased in

both animals following administration of the missing vitamin. The maximum change corresponded to formation of 44% instead of 58% of glucose from protein.

3. In dogs receiving a carbohydrate-free diet containing 72.2% of casein, pyridoxine deficiency appeared rapidly, and thiamine deficiency in the usual time, following withdrawal of the corresponding vitamin.

4. The disturbances in the central nervous system sometimes seen in dogs during pyridoxine deficiency were observed in one animal in the absence of hypoglycemia or anemia. The supply of glucose and oxygen to the nervous system is thus presumably normal, though the possibility of interference with utilization cannot be excluded.

5. Serum alkaline phosphatase was elevated during all phlorhizin periods in which it was determined. The relationship of this finding to changes in phosphatase observed in other forms of diabetes is discussed.

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THE THREONINE REQUIREMENT OF THE CHICK

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TWO FIGURES

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Threonine has been reported to be a dietary essential for rats (Rose, '38), mice (Bauer and Berg, '43), and chicks (Hegsted, '44; Almquist and Grau, '44). In his amino acid mixture, Hegsted used 1.8% DL-threonine; Almquist and Grau used levels of 1%, 2% and 3%, and found that while there was no difference in growth rate between 2% and 3% levels, 1% appeared insufficient for growth. Grau ('47) obtained satisfactory growth with an amino acid mixture which contained 1.3% DL-threonine. On the basis of the threonine contents of various proteins and the growth rates observed when these proteins were fed to chicks, Almquist ('47) has estimated the requirement to be about 0.6% L-threonine. Wilkening, Schweigert, Pearson and Sherwood ('47) found that 0.67% L-threonine from gelatin and oxidized casein apparently satisfied the chick's requirements.

In the experiments reported here, the aim was to determine the threonine requirements more accurately. To this end, groups of birds were fed diets containing various levels of threonine in which the nitrogen source was a mixture of crystalline amino acids.

METHODS

This report is based on the results obtained from 5 experiments performed over a two-year period. The amino acid

mixture used in the first experiment was identical with that used for the first experiment of a previously published report (Grau and Peterson, '46); a simpler mixture was used in subsequent experiments (table 1). The basal diet contained one

TABLE 1

Mixtures of amino acids used in the basal diets[1]

AMINO ACID	LEVEL IN THE DIET	
	Expt. 1	Expts. 2, 3, and 4
	%	%
DL-Alanine	1.5	1.5
L-Arginine HCl	1.4	1.4
DL-Aspartic acid	1.0	
L-Cystine	0.5	0.5
L-Glutamic acid	5.0	7.0
Glycine	1.8	1.8
L-Histidine HCl H_2O	0.8	0.8
L-Hydroxyproline	0.2	
DL-Isoleucine	2.0	1.0
L-Leucine	2.0	1.5
L-Lysine HCl H_2O	1.4	1.4
DL-Methionine	0.6	0.6
DL-Norleucine	0.2	
DL-Phenylalanine	1.5	1.0
L-Proline	2.0	
L-Tryptophane	0.3	
DL-Tryptophane		0.6
L-Tyrosine	2.0	1.0
DL-Valine	2.0	1.5

[1] All amino acids were commercial products of C.P. grade.

of the amino acid mixtures of table 1 plus the following ingredients in grams per 100 gm of diet: cellulose[1] 5.0, crude soybean oil 5.0, calcium gluconate 8.0, tricalcium phosphate 2.0, sodium dihydrogen phosphate[2] 1.0, potassium chloride[3]

[1] Cellu flour.

[2] In some experiments, dipotassium phosphate was used instead of sodium dihydrogen phosphate; however, the levels of potassium, phosphorus and sodium were kept constant by manipulation of the potassium chloride level (see table 2).

[3] See footnote 2.

0.6, sodium bicarbonate 1.5, choline chloride[4] 0.2, inositol[5] 0.1, cholic acid[6] 0.1, mixed tocopherols[7] 0.05, and fortified sardine oil (3000 A–400 D per gm) 0.25; also in milligrams per 100 gm of diet, the following: thiamine hydrochloride 1, riboflavin 1, pyridoxine hydrochloride 1, nicotinic acid 5, calcium(d)pantothenate 3, 2-methyl-1,4-naphthohydroquinone diacetate 1, biotin[8] 0.1, pteroylglutamic acid[9] 1, manganese 10, silicon 46, magnesium 48, aluminum 8, iron 14, copper 1, zinc 1, iodine 0.8, and cobalt 0.5. DL-Threonine or L-threonine from commercial sources was added in varying amounts and glucose[10] was added to give a total of 100 gm.

In all experiments except number 2a a conditioning, or pre-test, diet was employed for a few days prior to the start of the experiments in order to accustom the chicks to the consistency of the experimental diets. The pre-test diet was essentially the same as the experimental diet except that 20% crude casein and 10% gelatin replaced the mixture of pure amino acids.

As will be pointed out in some detail, poor growth rates were obtained in experiments 2a and 3, even at levels of threonine which had allowed satisfactory growth in previous tests. For this reason, it appeared desirable to summarize all the variable experimental conditions in tabular form. These data are presented in table 2.

The growth rates are expressed as per cent gain per day,[11] in order to allow comparisons among experiments despite

[4] Provided by Lederle Laboratories Division, American Cyanamid Co., through the courtesy of Dr. T. H. Jukes.

[5] Omitted from the diets used in experiments 3 and 4 (see table 2).

[6] See footnote 5.

[7] Concentrate of natural mixed tocopherols (15%), Distillation Products, Inc.

[8] Provided by Merck and Co., through the courtesy of Dr. D. F. Green.

[9] See footnote 4.

[10] Cerelose.

[11] $$\text{Per cent gain per day} = \frac{\text{Ave. gain during expt.} \times 100}{\text{Ave. weight during expt.} \times \text{no. days on expt.}}$$

slight differences in average starting weights and length of time of experiment. The actual gains can easily be calculated from the growth rates and the average weights at the start of the experiments (table 2).

TABLE 2

Known variables which were present in the several experiments

EXP. NO.	NO. OF DAYS ON EACH DIET		NO. OF CHICKS PER GROUP	WEIGHT AT START OF EXPERIMENT		NO. OF DAYS ON EXPERIMENT	AMINO ACID MIXTURE USED [1]	BASAL DIET USED [2]	GROWTH RESULTS WITH POSITIVE CONTROL DIET
	Chick mash	Pre-test		Average	Range				
				gm	*gm*				
1	14	3	4	93	85–100	10	old	old	good
2a	14	0	4	77	75–79	8	new	old	poor
2b	9	7	4	89	79–93	7	new	old	good
3	9	4	4	83	76–91	8	new	new	poor
4	9	5	3	92	86–98	7	new	new	good

[1] The differences between "old" and "new" amino acid mixtures are detailed in table 1.

[2] The "old" basal diet was exactly as presented under Methods; the "new" diet incorporated the changes stated in text footnotes 2 and 5, pages 106 and 107.

RESULTS

Experiment 1

Five groups of chicks fed diets which contained various levels of DL-threonine grew at the average rates shown in figure 1. The best growth rates observed here were as good as rates previously reported when chicks were fed mixtures of amino acids (Grau and Peterson, '46). From this experiment it was apparent: (1) that 0.5% DL-threonine is insufficient for satisfactory growth; and (2) that the requirement is certainly not much greater than 1% of the DL form.

Experiment 2a

The best growth obtained with chicks fed these diets was only about 1.5% per day, which is considerably poorer than in experiment 1. Since these chicks were not fed the condition-

ing diet prior to the experiment, it was felt at the time that the chicks received a setback when the experimental diet was first presented to them. The point of inflection of the growth curve was at approximately 1% DL-threonine (fig. 1).

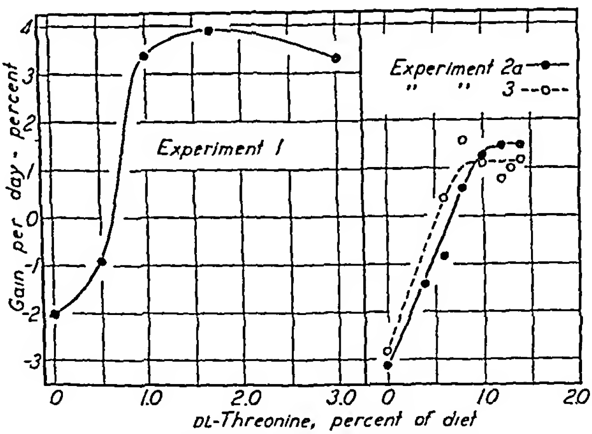

Fig. 1 The effect of feeding various levels of DL-threonine on the rate of growth. Each point represents the average growth rates of 4 chicks.

Experiment 2b

Since the reason for the poor results obtained in experiment 2a was not apparent, the following experiment was performed: The unused diet of experiment 2a was weighed, mixed, and divided into two portions, to one of which 0.5% DL-isoleucine, 0.5% DL-valine and 0.2% DL-methionine were added. The DL-threonine content of both diets was 1.28%. After being fed a chick mash for 9 days and the pre-test diet for 7 days, chicks were sorted into two groups and given these diets. For the chicks receiving the extra isoleucine, valine and methionine, the growth rate was 3.9% per day; for those not receiving these supplements, the rate was 3.5% per day. The difference between these rates cannot be considered significant. Both compared very well with those obtained in experiment 1. It was apparent, then, that the amino acid mixture per se was not at fault, and the reason for the poor results of experiment 2a remained obscure.

Experiment 3

This experiment was essentially a repetition of experiment 2a, designed to test the reproducibility of the results of that experiment. The poor growth rates at high threonine levels, together with the good growth rates of experiment 2b, necessitated a critical examination of the known variables before experiment 4 was undertaken. Without a pre-test period, poor results were obtained in experiment 2a, but a three-day pre-test period appeared satisfactory in experiment 1. A 4-day period was used in experiment 3, and poor results were obtained. Thus, the length of time of the pre-test diet period did not appear to be responsible for the varying growth rates obtained.

The age of the chicks at the start of the experiments (the sum of days in columns 2 and 3 of table 2) is another variable which might be responsible. Good results were obtained with ages of 17 days (experiment 1) and 16 days (experiment 2b), and poor results were obtained with 14 days (experiment 2a) and 13 days (experiment 3). These age differences caused differences in average starting weights, also, as shown in columns 5 and 6 of table 2. These data, together with the fact that the amino acid mixture and basal diet used had no significant effect on the results obtained, led to the tentative hypothesis that the smaller chicks were not able to adjust to the amino acid diet, even though they had been fed a highly purified pre-test diet. As a test of this hypothesis the largest chicks available were chosen for experiment 4, but their age at the start of the experiment was only 14 days.

Experiment 4

Good growth was obtained at the higher levels of threonine, as shown in figure 2. In this figure the individual growth rate of each chick is presented, in order to allow a critical evaluation of the effectiveness of the natural enantiomorph of threonine as compared with DL-threonine. Here the abscissae are expressed in terms of L-threonine. If both D- and L-threonine

were effective in promoting growth, twice as much L-threonine as DL-threonine would be required to result in a particular rate of growth when this rate is below the maximum growth rate. The results of this experiment indicate that the L-threonine requirement is certainly not lower than 0.4% and not higher than 0.5% of the diet, and that the D form is not utilized by the chick to any significant extent.

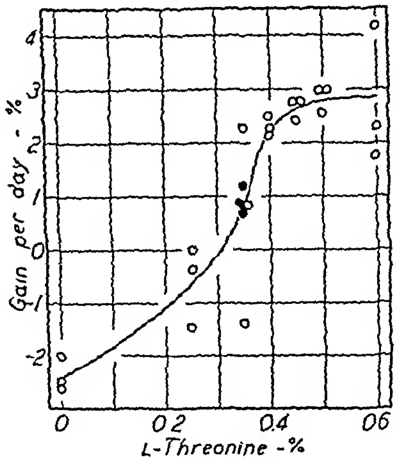

Fig. 2 The results of experiment 4. Each point represents a single chick. DL-Threonine was fed to all chicks denoted by open circles but only the L-threonine level is plotted here. The closed circles denote chicks fed L-threonine.

DISCUSSION

The results presented above must be considered from several aspects before the tentative threonine requirements are set. The first concerns the validity of using mixtures of amino acids to determine quantitative requirements, in view of the acknowledged fact that the best growth rate obtained when a mixture is fed is less than the best one obtained when intact proteins are fed. Since there appears to be considerable justification for considering the amino acid data valid (Almquist, '47), tentative standards may be set up on this basis; as the studies of amino acid requirements are extended and refined, however, differences between whole and hydro-

lyzed proteins may become significant and further revisions of requirements may be needed.

It is apparent from the results plotted in figure 2 that only the natural, or L, form of threonine is utilized by the chick under these conditions; hence the data of figure 1 should be considered only in terms of the levels of L-threonine which were present. These data indicate that 0.5% L-threonine is adequate, and that 0.3% is inadequate, for best growth. Two of the three experiments which allow a close evaluation of the requirement indicate that 0.45% is as good as 0.5%; hence it seems justifiable to set the tentative L-threonine requirement of growing chicks at 0.45% of the diet.

As was stated above, the poor results observed in experiments 2a and 3 can best be correlated with the fact that comparatively small chicks were used in these experiments. The reason for the poor acceptability of the diets is not clear, since other experiments, which have given satisfactory results, have been performed with chicks comparable to the small chicks used here (Grau, '47).

According to recent analyses of the threonine content of feedstuff proteins (Schweigert, '48), only the proteins of bone meal and certain types of wheat contain insufficient threonine to satisfy the chick requirement. These calculations are based on the fact that a protein which is used to provide 20% protein to the diet must contain 2.25% threonine $\left(\frac{100 \times 0.45}{20}\right)$ if the chick's requirement is to be met. Peanut meal protein is a marginal threonine source, but results of feeding trials have indicated that a 20% protein diet in which the sole amino acid source was peanut meal provided a satisfactory amount of threonine (Grau, '46).

SUMMARY

Highly purified diets in which mixtures of amino acids replaced proteins were used to determine the level of threonine required for growth by young White Leghorn chicks. Four experiments were performed in which various levels of DL-threonine were fed; in one experiment, L-threonine was compared to DL-threonine for growth-promoting activity. DL-

Threonine was found to be only half as active as L-threonine; hence the requirement is best expressed in terms of the L-form. Under these conditions, the L-threonine requirement was found to be approximately 0.45% of the diet.

In two of the experiments poor growth was obtained even at satisfactory levels of threonine. Although the cause for these poor results could not be ascertained unequivocally from the data, it is suggested that the use of small chicks in some experiments was an important factor.

A comparison of the threonine requirement with the threonine content of feedstuff proteins indicates that a deficiency of this amino acid under practical conditions is unlikely.

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THE EFFECT OF HEAT ON THE NUTRITIVE VALUE OF LACTALBUMIN

I. GROWTH ON DIETS CONTAINING HEATED PROTEINS [1]

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The detrimental effect of heat on the nutritive value of proteins was first reported by McCollum and Davis, in 1917, and has since been the subject of continued investigation. The growth-promoting property of casein, white and whole wheat bread, rice, corn, and wheat gluten (Morgan, '31), edestin (Waisman and Elvehjem, '38) and soybean meal (Bird and Burkhardt, '43) has been shown to decrease when the protein is toasted or autoclaved under various conditions.

Greaves, Morgan and Loveen ('38) have reported that in casein, lysine is the first amino acid rendered nutritionally unavailable when the protein is heated, and that histidine is next in order. These authors observed much improved growth in rats when they supplemented diets containing heated casein with the two amino acids. Lysine supplementation has also been shown to improve the growth-promoting property of autoclaved edestin (Waisman and Elvehjem, '38). On the other hand, Seegers and Mattill ('35), using beef protein, and Evans, McGinnis and St. John ('47), using soybean protein, have reported that heated protein is less susceptible to *in vitro* enzymatic digestion than is the unheated protein, and they have suggested that the alteration in digestibility

[1] The data in this paper are taken from the dissertation submitted by Ruth M. Davis for the degree of Doctor of Philosophy, Wayne University, 1948.

may be a major factor in the change in nutritive value of the proteins concerned.

The present report is based on a study of the nutritive value of heated lactalbumin with emphasis on the effect of varying the temperature used, the duration of the heating process, and the methods of heating. The value of lysine supplementation of diets containing heated lactalbumin, the effect of heat on the essential amino acids themselves, and the digestibility of heated lactalbumin have likewise been studied.

EXPERIMENTAL

Plan

Male albino rats approximately 25 days old and weighing between 40 and 60 gm were housed in individual cages with mesh bottoms designed to prevent coprophagy. Body weight changes and food intakes were recorded regularly. The basal diet contained 16.6 mg of nitrogen per gram of diet when lactalbumin was used (or 12–13.5% by weight of the heated protein), 51.2–49.7% dextrin, 12.8% sucrose, 20% corn oil,[2] and 4% salt mixture (Wesson, '32). The vitamin supplement incorporated into all the rations is shown in table 1.

The lactalbumin used in these experiments was a commercial product.[3] In some of the experiments it was baked in shallow layers approximately 5 mm deep at a constant temperature for one hour, and in other experiments the protein was autoclaved in similar layers at 116–120°C. under 15 lbs. pressure for 15, 30, or 60 minutes. The various heat treatments accorded the proteins are summarized in table 1. The color of the protein deepens progressively as the temperature increases when the protein is dry heated. However, when the protein is autoclaved at 120° for one hour the color is much darker than when it is dry heated at 140° for one hour, although not as dark as when it is dry heated at 200° for the same period. In fact, there appears to be a direct re-

[2] Mazola.

[3] Lactalbumin 15–42, obtained from the Borden Company, New York.

TABLE 1

Diets: treatment of protein, and composition

TREATMENT OF PROTEIN	DESIGNATION OF DIET [1]	VITAMIN SUPPLEMENTS PER KG OF DIET	
Lactalbumin, unheated	UnL	Thiamine HCl [3]	10 mg
Lactalbumin, dry heated for 1 hour at 140°C.	140L–60D	Riboflavin [3]	20 mg
Lactalbumin, dry heated for 1 hour at 120°C.	120L–60D	Pyridoxine HCl [4]	10 mg
Lactalbumin, dry heated for 1 hour at 200°C.	200L–60D	Niacinamide [4]	20 mg
Lactalbumin, autoclaved for 1 hour at 120°C.	120L–60A	Calcium pantothenate [4]	40 mg
Lactalbumin, autoclaved for 30 min. at 120°C.	120L–30A	Choline [4]	2000 mg
Lactalbumin, autoclaved for 15 min. at 120°C.	120L–15A	Inositol [4]	2000 mg
Gliadin, unheated	GL	*p*-Aminobenzoic acid [4]	600 mg
		Biotin [4]	10 μg
		2-Methylnaphthoquinone [4]	4 mg
		α-Tocopherol [4]	30 mg
		Folic acid [5]	20 mg
		Vitamin A [2,6]	
		Vitamin D [2,6]	

[1] The abbreviations designating the diets refer to the protein component: "L" denotes lactalbumin; 120, 140, and 200 refer to the temperature used; 15, 30, and 60 refer to the duration of heating in minutes. The letter "D" shows that the protein was heated in a dry oven, while "A" represents the autoclaved protein.

[2] Courtesy of Parke, Davis and Company.

[3] Courtesy of Charles Pfizer and Company.

[4] Courtesy of Merck and Company.

[5] Courtesy of Lederle Laboratories.

[6] Four thousand units of vitamin A and 800 units of vitamin D were given per week, as Natola.

lationship between the color developed during the heating process and the nutritive value of the protein. Weast, Groody and Morgan ('48) observed similar color changes in their dry heated casein.

Growth

Twenty rats were maintained on each of 4 dietary regimes: UnL, 120L–60A, 140L–60D, and 200L–60D (see footnote 1, table 1). Inasmuch as the animals given diet 200L–60D daily

TABLE 2

Comparison of growth obtained under conditions of restricted food intake and ad libitum feeding

DIET [1]		DAYS ON DIET	WEIGHT CHANGE	DAILY FOOD INTAKE	WEIGHT CHANGE PER GM FOOD EATEN
			gm	*gm*	*mg*
		Average body weight changes — restricted intake			
UnL	(20)[2]	21	+ 4.90	3.2	+ 59
140L–60D	(19)	21	+ 2.77	3.2	+ 36
120L–60A	(20)	21	— 0.57	3.2	— 10
200L–60D	(19)	21	— 15.45	3.2	—233
		Average body weight changes — ad libitum			
UnL	(5)	56	+ 132.4	7.8	+ 232
140L–60D	(10)	56	+ 27.2	5.6	+ 69
120L–60A	(5)	32	— 10.4	5.1	— 66
200L–60D	(5)	18	— 14.9	4.5	—227

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

consumed the least food, the rats on the other diets were pair-fed with respect to this group. The animals given diet 200L–60D rapidly lost weight, whereas those given diet 120L–60A almost maintained their starting weight and those on diet 140L–60D were able to make a slight gain in weight, although they did not grow as well as did the control animals (table 2). When another series of animals was allowed the same diets ad libitum the differences in the growth-promoting abilities

of these various diets were more apparent but in the same order (table 2). Since the rats reacted to environmental conditions such as temperature and humidity, animals used during the summer did not grow as well as the others. Therefore control animals were used with the spring, summer, and fall groups and the experiments on diets 120L–60A, 140L–60D, 200L–60D were repeated at these periods, with essentially the same results.

Amino acid supplementation

Because of previous reports of the effectiveness of lysine and histidine in restoring the growth-promoting ability of heated proteins (Greaves, Morgan and Loveen, '38; Waisman and Elvehjem, '38), these amino acids were used as supplements with heated lactalbumin. Nine and six-tenths grams of lysine monohydrochloride were added to each kilogram of diet 200L–60D in preparing diet 200L–60D–Ly, and 3 gm of histidine monohydrochloride added to diet 200L–60D–Ly gave diet 200L–60D–LyH. Neither lysine nor lysine plus histidine restored the growth-promoting capacity of diets containing lactalbumin which had been baked at 200°C. for one hour (table 3).

The progressive nature of the changes produced in the protein by heating at various temperatures, as shown in table 2, suggested that lysine supplementation of lactalbumin might be efficacious with diets 120L–60A and 140L–60D. Table 3 shows that growth rates of the rats fed lysine supplements did not approach those of the control animals. Rats that had been on a restricted intake of diets UnL, 140–60D, or 120L–60A for three weeks were allowed to eat ad libitum for the following 8 weeks. One animal on the UnL diet, three rats on diet 120L–60A, and 4 rats on diet 140L–60D received the same diet that they had received for the first three weeks. Four others previously on diet 120L–60A and 4 on diet 140L–60D were given the lysine supplement (9.6 gm per kilogram of diet). These animals consumed more food and gained more weight than rats fed similar diets from weaning and

without the prior restricted intake period. However, the lysine supplement was only slightly beneficial with diet 140L–60D and of no value with diet 120L–60A. The control animal exhibited the same acceleration in growth rate during this period as was seen in the others but gained significantly more weight per gram of food consumed than the rats fed

TABLE 3

Effect of supplementing heated lactalbumin and gliadin with lysine, or histine, or both

Average body weight changes

DIET [1]	DAYS ON DIET	WEIGHT CHANGE	DAILY FOOD INTAKE	WEIGHT CHANGE PER GM FOOD EATEN
		gm	*gm*	*mg*
200L–60D (15)[2]	21	— 15.8	3.8	—193
200L–60D–Ly (5)	21	— 15.4	3.7	—199
200L–60D–LyH (5)	21	— 14.7	3.8	—183
140L–60D (4)	56	+ 82.9	8.1	+180
140L–60D–Ly (4)	56	+106.6	8.4	+224
120L–60A (3)	56	+ 35.3	5.0	+118
120L–60A–Ly (4)	56	+ 32.3	4.7	+122
UnL (1)	56	+188.0	9.3	+355
140L–60D (10)	56	+ 27.1	6.4	+ 69
140L–60D–3Ly (10)	56	+ 23.0	6.0	+ 59
UnL	56	+132.4	7.8	+232
GL (10)	56	— 9.4	4.1	— 41
Gl–Ly (10)	56	+ 18.2	5.1	+ 60
Gl–140Ly (10)	56	+ 17.8	5.5	+ 57

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

the heated protein. To insure adequate supplementation the amount of lysine used in diet 140L–60D–Ly was tripled and another series of rats was given this diet (140L–60D–3Ly). The supplementation produced no improvement in the growth of these animals (table 3) and it can be concluded that the decrease in the nutritive value of heated lactalbumin is not primarily due to the unavailability of lysine to the animal.

Further evidence that heat does not injure the lysine fraction per se was obtained by heating lysine monohydrochloride at 140°C. for one hour in a dry oven and comparing the ability of the heated (140Ly) and unheated (Ly) amino acid to promote growth in rats maintained on diets in which gliadin was the sole source of protein (table 3). No differences were observed in the growth rates of the animals receiving the two supplements; the rats apparently were able to utilize the heated as efficiently as the unheated amino acid.

TABLE 4

Growth on diets with and without heated amino acids

Average body weight changes

DIET	DAYS ON DIET	WEIGHT CHANGE	DAILY FOOD INTAKE	WEIGHT CHANGE PER GM FOOD EATEN
		gm	*gm*	*mg*
AAH (10)[1]	35	+ 53.0	8.1	+ 188
AA (8)[2]	35	+ 44.0	7.7	+ 167

[1] Figures in parentheses denote number of rats used.
AAH signifies amino acid mixture, heated.

[2] Signifies amino acid mixture, unheated.

Greenhut et al. ('48) have shown that there is no loss of lysine, threonine or methionine in the process of roasting meat. However, there have been no reports in the literature upon the effect of heat on a mixture of the 10 essential amino acids; therefore, such a mixture[4] was prepared (according to Rose, '38) and heated dry at 140°C. in an oven for one hour. The mixture, both heated (AAH) and unheated (AA), constituted 12% of the total diet. Under these conditions none of the essential amino acids was so changed by heat as to render it unavailable to the animal for growth, as compared with the growth obtained by the rats fed the same mixture unheated (table 4). In contrast, the nutritive value of lactalbumin is considerably decreased by such treatment.

[4] Courtesy of Frederick Stearns and Company.

Digestibility

Observations on the animals fed diet 200L–60D suggested that these animals received no benefit from the protein component of the ration. To test this theory, 5 rats were fed a diet containing no protein (diet NP) but otherwise identical with diet 200L–60D, with dextrin replacing the protein isocalorically. The rats on diet NP lost almost the same amount of weight as those on diet 200L–60D (15.4 gm and 15.8 gm, respectively) and at the same rate, over a three-week period, and consumed 3.7 gm of diet daily as compared to the 3.8 gm daily consumed by the 200L–60D group. The weight change per gram of food consumed by rats on diets NP and 200L–60D was —206 and —202 mg, respectively.

In order to ascertain whether the lowered nutritive value of the heated protein was the result of decreased digestion and/or absorption, or whether it resulted from non-utilization of the absorbed amino acid, a comparative study was made of the fecal nitrogen excretion of rats maintained on diets 140L–60D, 120L–60A, NP, UnL, and Tox (diet UnL to which 60 gm of lactalbumin which had been heated at 200°C. for one hour was added per kilogram of diet). Feces were collected from individual rats over 24-hour periods after the animals had consumed their respective rations for at least one week, and the total 24-hour collection was analyzed for total nitrogen. The average amount of fecal nitrogen excreted by the rats on diet NP was considered the basal, or endogenous, nitrogen, and this quantity was subtracted from the amount of nitrogen excreted by the animals on protein-containing diets. The rats on diet NP excreted 2.33 mg of nitrogen per gram of food consumed, or an average of 8.3 mg of nitrogen per 24 hours. The fecal nitrogen excretion of the rats on all the diets containing heated protein was higher than was that of the control animals (table 5).

The suggestion that the lowered nutritive value of heated lactalbumin was the result of a decreased digestibility of the protein was further supported by an *in vitro* study. A quan-

tity of the protein equivalent to 0.55 gm of nitrogen was incubated with pepsin (pH 1.5) and then pancreatin (pH 8.0) at 37°C. for a total of 7 days, and the mg of nitrogen present in an aliqout of the supernatant fluid determined by Sorenson's formol titration procedure. As can be seen in table 5, the amount of digestion occurring *in vitro* correlates well with the ability of the rats to grow on the various diets. It appears, therefore, that the change in the digestibility of the protein after heating is a significant factor in the decrease in its nutritive value.

TABLE 5

Digestibility of heated protein — in vivo and in vitro observations

IN VIVO		IN VITRO	
Diet[1]	N excreted per gm food consumed	Protein	N in supernatant fluid after in vitro digestion
	mg		*mg %*
UnL (10)[2]	2.49 ± 0.24[3]	UnL	120.0
140L–60D (10)	7.77 ± 0.83	140L–60D	82.9
120L–60A (8)	6.18 ± 0.97	120L–60A	34.7
Tox (10)	8.43 ± 0.90	200L–60D	4.1

[1] For meaning of symbols, see table 1.

[2] Figures in parentheses denote number of rats used.

[3] Standard error.

Methods of heating

In addition to varying the temperature used during the heating process, the effect of varying the methods of heating and the duration of heating was studied. Rats maintained on diets containing lactalbumin which had been autoclaved at 120°C. (15 lb. pressure) for one hour (diet 120L–60A) lost an average of 10.4 gm in 32 days, whereas those animals fed lactalbumin which had been oven-baked at 120°C. for one hour (diet 120L–60D) gained an average of 56.8 gm in that period. Although the rats on diet 120L–60D gained slightly less weight than did the controls (56.8 gm and 68.4 gm respectively), it is obvious that the decrease in the nutritive value of lactalbumin is considerably greater when the protein

has been autoclaved at 120°C. than when it has been heated in a dry oven at the same temperature. The average daily food intakes of the animals fed diets 120L–60A, 120L–60D, and UnL were 5.1 gm, 9.5 gm, and 9.6 gm respectively, and their respective weight changes per gram of food consumed were —66 mg, + 187 mg and + 223 mg.

Table 6 shows that autoclaving at the same temperature and pressure (120°, 15 lb.) for 15 or 30 minutes (Diets 120L–15A and 120L–30A) reduces the nutritive value of lactalbumin

TABLE 6

Influence of duration of heating

Average changes in body weight

DIET [1]	DAYS ON DIET	WEIGHT CHANGE	DAILY FOOD INTAKE	WEIGHT CHANGE PER GM FOOD EATEN
		gm	*gm*	*mg*
UnL (5)[2]	56	+ 132.4	7.8	+ 232
120L–15A (9)	56	+ 41.5	7.4	+ 100
120L–30A (10)	56	+ 11.3	6.6	+ 25
120L–60A (5)	32	— 10.4	5.1	— 66

[1]For meaning of symbols, see table 1.

[2] Numbers in parentheses denote number of rats used.

to a considerable degree. Because of the wide use of the pressure cooker in the American household, the authors believe that these findings warrant further investigation.

The animals used in the various experiments were autopsied and complete histological examinations were made. The results will be reported in a later communication.

DISCUSSION

The data presented above show that the nutritive value of lactalbumin is decreased progressively when the protein is heated. The temperature, duration of heating, and method of heating all are factors which determine the amount of damage sustained by the protein. Pressure and/or moisture

accelerates the production of the deleterious change which occurs in lactalbumin when it is heated.

Contrary to the results obtained with casein by Morgan ('31) and with edestin by Waisman and Elvehjem ('38), the addition of lysine to diets containing heated lactalbumin did not significantly improve their biological value, and it can be concluded that the decreased nutritive value of lactalbumin after heating is not primarily the result of a change in the lysine fraction. There was no evidence of a toxic factor in the heated protein. The change produced in this protein by heat does considerably decrease the digestibility of the protein, to the extent that when the protein has been heated at 200°C. for one hour it is practically indigestible. Although Greaves, Morgan and Loveen ('38) using rats as the experimental animal, reported that the decrease in the digestibility of heated casein was not sufficient to explain the decrease in the biological value of the same protein, this does not appear to be the case with heated lactalbumin. Evans, McGinnis and St. John ('47), using chicks as the experimental animal, have found that the digestibility of over-cooked soybean meal is also decreased. In a recent communication Weast, Groody and Morgan ('48) reported that as much as 94–96% of the nitrogen excreted by dogs fed casein heated at 200° appeared in the feces. We have found that the nitrogen of heated lactalbumin is similarly not absorbed by the rat.

In view of the fact that heating (140°C.) the indispensable amino acids does not decrease their utilization, as shown by comparative growth studies, the change produced in a protein by heat probably does not affect the structure of the amino acid per se. The site of the alteration in heated lactalbumin appears to be at the linkages between the amino acids; either the peptide linkages or the grid linkages or both are so changed by heat as to become resistant to enzymatic hydrolysis. The progressive nature of this change may indicate that certain linkages are more susceptible to change by heating than others.

SUMMARY

The nutritive value of lactalbumin is diminished by heating the protein. This decrease appears to be the result of lowered digestibility of the heated protein due to a change in the linkages between the amino acids which renders them less susceptible to enzymatic hydrolysis. This change is produced at lower temperatures and in less time when the protein is autoclaved than when it is baked in a dry oven.

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THE EFFECT OF PTEROYLGLUTAMIC ACID AND AN UNIDENTIFIED FACTOR ON EGG PRODUCTION AND HATCHABILITY[1]

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Bauernfeind and Norris ('39) and Schumacher, Heuser and Norris ('40) reported a factor necessary for breeding hens which they designated Factor R and which subsequently has been suggested to be a mixture of folic acid conjugates (Charkey, Daniel, Farmer, Norris and Heuser, '47). The essential nature of a factor present in the *Lactobacillus casei* ε eluate fraction of solubilized liver extract for breeding hens was established by Cravens, Sebesta, Halpin and Hart ('42). The identification of the *L. casei* factor as pteroylglutamic acid was established by Angier et al. ('46). Since synthetic pteroylglutamic acid (PGA) has become available, Taylor ('47) and Schweigert, German, Pearson and Sherwood ('48) have published reports on the quantitative needs of the breeding hen for this vitamin. Taylor ('47) reported that 0.12 mg per kilogram of ration was essential for egg production, while an amount somewhat in excess of this was required for satisfactory hatchability. Schweigert et al. ('48), on the other hand, reported that the requirement is between 0.12 and 0.42 mg per kilogram of ration.

During the course of studies designed to determine the effect of pteroylglutamic acid on egg production and hatch-

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This study was supported in part by a grant from the International Baby Chick Association, Kansas City, Missouri.

ability it became obvious that the results were complicated by a deficiency of an unidentified factor or factors. Data will be presented in this report indicating the importance of such a factor or factors for hatchability. Further, some of the data may shed some light on the quantitative needs of the breeding hen for pteroylglutamic acid.

EXPERIMENTAL

Single Comb White Leghorn pullets were used in all experiments. The birds were housed in individual-cage laying-batteries with raised screened floors. They were artificially

TABLE 1

Composition of experimental diets

INGREDIENTS	BASAL DIETS				VITAMINS, CHOLINE AND INOSITOL ADDED TO ALL BASAL DIETS	
	B20	B22	B23	B24		
	%	%	%	%		mg/kg of diet
Dextrin	64	67			Riboflavin	6.0
Sucrose			67	67	Thiamine-HCl	4.0
Casein [1]	18	18	18	18	Ca pantothenate	15.0
Gelatin	5	5	5	5	Niacin	100
Salts IV	5	5	5	5	Pyridoxine-HCl	4.0
Soybean oil	3	3	3	3	2-Methyl 1,4-	
Vitamins A and D					naphthoquinone	0.5
(400D–3000A)	2	2	2	2	Biotin	0.2
Liver fraction L	3.0				Alpha-tocopherol	3.0
Fish solubles					Choline	2,000
(50% solids)				3.0	Inositol	1,000

[1] Vitamin test. General Biochemicals, Inc.

inseminated weekly with pooled semen from New Hampshire males. All birds were weighed weekly. Eggs were collected daily, marked with the hen's number and recorded. Settings of eggs were made weekly. All eggs failing to hatch were broken to determine fertility and to make gross examinations of the embryos for developmental abnormalities.

The composition of the basal diets used is given in table 1. Feed, water and oyster shell were supplied ad libitum.

Experiment 1 was designed to obtain information on the pteroylglutamic acid requirements of the breeding chicken. Five groups of 4 birds each were fed the following rations for 11 weeks: Group 1, diet B20 (which served as a control since this diet had proved satisfactory in previous experiments); group 2, diet B22; groups 3–5, ration B22 with pteroylglutamic acid additions at levels of 0.1, 0.25 and 1.0 mg per kilogram of diet, respectively. The hatchability and egg production data are presented in table 2.

Experiment 2 was similar in design to experiment 1. The following diets were fed to 6 groups of three birds each for 14 weeks: Group 1, diet B20; group 2, B22; and groups 3–6,

TABLE 2

Effect of pteroylglutamic acid on egg production and hatchability

PGA SUPPLEMENT TO DIET B22:	EXPERIMENT 1		EXPERIMENT 2	
	Egg production	Hatchability	Egg production	Hatchability
mg/kg	%	%	%	%
0	54	40	36	32
0.10	59	56	40	35
0.20			39	28
0.25	61	51		
0.50			31	60
1.0	51	66	51	20
3% Liver L	57	83	55	71

diet B22 supplemented with 0.1, 0.2, 0.5 and 1.0 mg of pteroylglutamic acid per kilogram of diet, respectively. The hatchability and egg production data are given in table 2.

It is obvious from the results shown in table 2 that none of the diets fed in experiments 1 and 2, except perhaps the control, was adequate for good hatchability. It is also obvious that the response of the birds to the same diets in the two experiments was radically different. The possible significance of this observation will be discussed later, but suffice it to point out here that the results of experiments 1 and 2 show definitely that some unidentified factor, or factors, needed by breeding hens is missing from diet B22 supplemented with

PGA. It was also suspected that intestinal synthesis on the dextrin rations used in these experiments might be one factor responsible for certain of the erratic results observed, since Couch et al. ('48a) have shown that dextrin promotes intestinal synthesis of biotin in the mature fowl. Thus an experiment was designed to take these factors into consideration.

In the third experiment sucrose was compared with dextrin as a carbohydrate source and condensed fish solubles was tested as a source of unidentified factors. Additional purified casein was also added to eliminate the possibility that insufficient protein or amino acids might be affecting the results. The diets fed and the resulting egg production and hatchability for experiment 3 may be seen in table 3.

TABLE 3

Rations fed and per cent of egg production and hatchability (Experiment 3)

GROUP [1]	BASAL DIET	SUPPLEMENT TO BASAL DIET	EGG PRODUCTION	HATCHABILITY		
				Start of exp.	End of exp.	Ave.
			%	%	%	%
1	B22	None	59	75	29	62
2	B23	None	36	90	0.0	37
3	B22	4.0 mg per kg PGA	62	100	40	72
4	B23	4.0 mg per kg PGA	[2]	74	8.0	65
5	B22	6% casein [3]	51	87	17	45
6	B22	6% casein plus 4.0 mg per kg PGA	79	100	29	73
7	B23	6% casein plus 4.0 mg per kg PGA	61	96	20	76
8	B22	3% liver fraction L	82	75	91	83
9	B23	3% liver fraction L	75	89	88	84
10	B23	3% liver fraction L plus 6% casein	76	74	87	94
11	B22	4.0 mg per kg PGA plus 3% fish solubles	67	75	90	92
12	B23	same as group 11	76	90	71	86
13	Practical ration		68	95	95	93

[1] Four birds in each group.

[2] Not presented because of loss of two birds in this group.

[3] Vitamin test casein. General Biochemicals, Inc.

From the data shown in this table it appeared that satisfactory egg production and hatchability could be obtained using either sucrose or dextrin as a source of carbohydrate when liver fraction L or fish solubles and 4.0 mg of pteroylglutamic acid per kilogram of ration were included in the diets. With the results of experiment 3 at hand it appeared that a diet was available (group 12 of experiment 3) which

TABLE 4

Rations fed and per cent of egg production and hatchability (Experiment 4)

GROUP [1]	BASAL DIET	SUPPLEMENT TO BASAL DIET	EGG PRODUCTION	HATCHABILITY Start of exp.	HATCHABILITY End of exp.	HATCHABILITY Ave.
			%	%	%	%
2	B23	4.0 mg per kg PGA	48.5	75	0	27
3	B22	Same as group 2	70.8	86	36	54
4	B24	None	50.9	89	0	26
5	B24	0.25 mg per kg PGA	74.7	90	0	46
6	B24	0.50 mg per kg PGA	70.5	95	0	65
7	B24	0.75 mg per kg PGA	73.8	100	0	53
8	B24	1.0 mg per kg PGA	69.9	89	0	75
9	B24	4.0 mg per kg PGA	62.8	100	40	76
10	B22	3% fish solubles	55.7	87	31	53
11	B22	Same as group 10 plus 4.0 mg per kg PGA	59.5	95	36	80
12	B23 less gelatin	Same as group 2	43.9	100	0	41
13	Practical control		55.1	100	67	96

[1] Four birds in each group.

would make possible studies on the quantitative needs of breeding chickens for pteroylglutamic acid. Experiment 4 was then set up to gain evidence on this point and to obtain further evidence on the unidentified factor as well as the possible effect of the source of carbohydrate on the reaction of the birds to the diets used. The diets fed and the egg production and hatchability data for experiment 4 are given in table 4.

RESULTS AND DISCUSSION

Unidentified factors

The results of experiments 1 and 2, given in table 2, are of interest because they emphasize the need of breeding hens for some unidentified factor which is essential for embryonic development. Further, the data indicate that the bird may be depleted of this factor in a relatively short period of time. It was mentioned previously that a marked difference in response of the birds to identical rations in experiments 1 and 2 was observed. In experiment 2 the birds depleted much more quickly and uniformly than in experiment 1. In experiment 2 hatchability of all groups dropped to zero by the 7th week, while in experiment 1 only two groups dropped to zero by the 6th week and even in these hatchability again improved as the experiment progressed.

Data which suggest that diets containing dextrin may promote the synthesis of an unidentified factor or factors in the intestinal tract are given in tables 3 and 4. A comparison of the hatchability of the eggs from groups 3 and 4 of experiment 3 (table 3) shows that those of group 3 which were produced by hens receiving dextrin as a carbohydrate hatched better than those from hens receiving sucrose as a carbohydrate. The results of experiment 4, given in table 4, further indicate that dextrin stimulates the intestinal synthesis of an unidentified factor or factors essential for the breeding hen. The hens of group 3, which received dextrin as a carbohydrate, produced eggs which hatched significantly better than those from hens of group 2, which were receiving sucrose as a carbohydrate.

Further evidence showing the importance in the diet of breeding hens of an unidentified factor in fish solubles and liver fraction L is given in the results of experiments 3 and 4, presented in tables 3 and 4. In every case a rapid decline (to less than 20% in 4 weeks) in hatchability occurred when a sucrose diet containing no fish solubles or liver fraction L, but containing 4.0 mg per kilogram of pteroylglutamic acid

was fed to the birds. The drop in hatchability was prevented by the addition of either fish solubles or liver fraction L. Two birds of group 4 were discarded at 4 weeks as they had lost considerable weight and had developed anorexia. The injection of 0.1 ml of reticulogen [2] brought about a dramatic recovery, but obviously the two birds had to be eliminated from the group.

The addition of casein did not improve hatchability. It is interesting to note, however, that the addition of 6% purified casein (group 5) appeared to result in a more rapid depletion of the birds (to 13% in 4 weeks) and that this effect was apparently overcome by the addition of pteroylglutamic acid (group 6). The birds of group 12 which received the same diet as group 2, except that gelatin was omitted, did not deplete any more rapidly than those of group 2.

There is some evidence from these experiments that a deficiency of the factor found in fish solubles and liver fraction L resulted in a decline in egg production. For example, group 4 of experiment 3 produced eggs at a very low rate and, as mentioned previously, two of the birds in this group showed a pronounced decline in weight. By comparing the rate of production of the groups of birds receiving dextrin and no fish solubles with that of the groups receiving a similar diet containing sucrose (tables 3 and 4), it may be seen that the dextrin-fed birds produced at a higher rate, as would be expected if intestinal synthesis made available to the bird a limited quantity of the factor yet one sufficient to support egg production. It is believed, however, that additional data are necessary to demonstrate the possible effect of this factor on egg production, since with such great individual variations, the numbers involved in the present experiments are inadequate to determine significance.

The studies reported herein dealing with an unidentified factor or factors essential for the breeding bird are of interest chiefly because they demonstrate that the laying bird can be depleted very quickly of an unidentified factor es-

[2] Lilly.

sential for hatchability. Numerous reports dealing with unidentified factors essential for breeding hens have appeared, including those of McGinnis et al. ('44), Bird et al. ('46), Bethke et al. ('46), and Cravens et al. ('46). Rubin et al. ('47) presented data which indicated that 15–16 weeks of feeding all-vegetable diets deficient in an unidentified factor were required to deplete the birds. Rubin, Bird and Rothchild ('46) suggested that intestinal synthesis of an unidentified factor might occur in the mature bird since hen feces were found to be a good source of unidentified chick growth factors essential in all-vegetable chick rations. McGinnis, Stevens and Groves ('47) presented evidence which they interpreted as indicating that the growth factors were synthesized, for the most part, by microorganisms after the feces were voided and not in the intestinal tract of the hen. Similar results have been reported by Kennard et al. ('48). The data presented herein are in agreement with the work of Rubin, Bird and Rothchild ('46), and these data further suggest that such synthesis may be, in part, responsible for the prolonged depletion period required when diets are employed which promote intestinal synthesis. It is probable, however, that other unrecognized conditions affect the rate of depletion since, even with the purified diets employed in the present study, considerable variation in degree and time of depletion was observed. It seems logical to conclude, nevertheless, that rapid depletion may be obtained if conditions are right, since there is abundant evidence that the hen may be quickly depleted of water soluble vitamins, and the unidentified factor, or factors, from cow manure, fish solubles, fish meal and certain liver fractions is water soluble.

Pteroylglutamic acid

The results of experiment 4, given in table 4, fail to provide clear-cut data on the pteroylglutamic acid requirements of the breeding hen. It would appear from these data that between 0.5–1.0 mg per kilogram of diet is essential for good hatchability, but even at a level of 4.0 mg per kilogram hatchability was not maintained at a high level for the 12-week

experiment. A rapid falling off in hatchability had occurred on the 0.25, 0.50 and 0.75 mg levels by the 9th week of the experiment, while a similar fall in hatchability occurred at the 10th and 11th weeks for the 1.0 and 4.0 mg levels, respectively. The significance of these results is not apparent at this time and the possibility exists that the fall in hatchability at 11 and 12 weeks is related to some environmental conditions, since the control group (13) dropped to 67% during the last week of the experiment. Of course, the possibility also exists that the birds had become depleted of a second factor by this time, or that 3% fish solubles supplied inadequate quantities of the factor to support hatchability. It is obvious, however, that further experiments are necessary to determine the reason for the pronounced drop in hatchability observed during the latter phases of experiment 4.

There is no question but that the birds in group 4, experiment 4, produced eggs at a significantly lower rate than did the birds receiving an adequate amount of pteroylglutamic acid. The average production of the birds of group 4, given in table 4, does not give a true picture of the egg production data of this group, since there was a gradual decline in egg production to 11% by the end of the experiment. The drop in production was most rapid after the 8th week. No such decline was observed in group 5, which received 0.25 mg of pteroylglutamic acid per kilogram of diet. Neither was there a pronounced decline in the production of group 10, which was fed a diet deficient in pteroylglutamic acid with dextrin as the carbohydrate. These results suggest that 0.25 mg of this vitamin per kilogram of ration is adequate for egg production and, further, that with the rations containing carbohydrates that promote intestinal synthesis pteroylglutamic acid may be synthesized in sufficient quantities to meet the needs of the laying bird.

The results of this study are not in agreement with the results reported by Schweigert et al. ('48) regarding the pteroylglutamic acid requirements of the breeding bird. It appears likely that the higher requirement indicated in the

present study (0.5–1.0 mg per kilogram) results from the use of a type of basal diet which does not favor intestinal synthesis of the vitamin. That intestinal synthesis was a factor affecting hatchability may be seen by comparing the results of groups 4 (sucrose) and 10 (dextrin), table 4. Hatchability of eggs from the birds of group 10 declined rapidly, but after an apparent adjustment period it again increased, only to decline again. Such variations have been noted in studies on the effects of various carbohydrate sources on the intestinal synthesis of biotin (Couch et al., '48b). The diets employed by Schweigert et al. would appear to favor such synthesis and thus might lead to the suggestion of requirements far below the true optimum.

SUMMARY

1. Four experiments with breeding chickens have been described which demonstrate the importance for hatchability of an unidentified factor or factors.

2. Fish solubles and liver fraction L are good sources of the factor or factors.

3. The breeding hen may be depleted of the factor in from 4–6 weeks when purified diets are used.

4. Data are presented which indicate that dextrin favors the intestinal synthesis of the factor in the mature fowl.

5. The pteroylglutamic acid requirement of the laying bird is probably not over 0.25 mg per kilogram of diet.

6. The pteroylglutamic acid requirement of the breeding bird appears to be approximately 0.5–1.0 mg per kilogram of diet when a diet is employed which does not favor intestinal synthesis of the vitamin.

7. Evidence is presented which indicates that pteroylglutamic acid synthesis in the intestinal tract of the breeding hen is favored by diets containing dextrin as a carbohydrate.

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COMPARISON OF CHEMICAL ANALYSIS AND BIO-ASSAY AS MEASURES OF VITAMIN A VALUE OF SOME VEGETABLES AND THE EFFECT OF COMMINUTION UPON THE BIO-ASSAY VALUE

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Vegetables and fruits have been estimated to supply about two-thirds of the vitamin A value of the food consumed in the United States (Clark, Friend and Burk, '47); the green leafy and yellow vegetables (including sweet potatoes), which alone furnish about half of the total, are the most important single group of contributors. These calculations were based on figures in which weight was given to chemical measurements of carotene content as well as to biological measurements of vitamin A value reported in the literature. However, the carotene content of certain vegetables and fruits, particularly the yellow ones, measured by chemical means, has been shown to be an unreliable index of their actual vitamin A value. Graves ('42) says that when the results are to be "related to nutritional problems, the danger of relying on carotene estimations even as an index of the comparative activity of the yellow and orange vegetables can hardly be over-estimated."

Conversion factors for different vegetables, calculated from strictly parallel chemical and biological assays, would make it

possible to adapt results of the more economical chemical measurements of carotene content for use in dietary calculations. Such assays are also necessary to ascertain what factors other than the nature of the vegetable itself may influence, either favorably or adversely, the availability of carotene. Smith and Otis ('41, '43) demonstrated that there is great variation in both rat growth and vitamin A liver stores promoted by the same amount of carotene, depending upon the vegetable source, and observed that "yellow foods are especially inefficient in promoting vitamin A reserves." Their data, however, do not lend themselves to quantitative interpretation because of the methods employed. According to experiments reported by Fraps and Meinke ('45), differences exist between vegetables and carotene in oil solution and among the vegetables themselves with respect to the availability of the carotene for accumulation of vitamin A in the liver; Kemmerer and Fraps ('45) were unable to find such differences, however, when the suboptimum rat-growth rate employed in bioassays was the criterion. Wilson et al. ('42, '46) have reported good agreement between chemical and rat growth assays of the vitamin A value of fresh carrots and of fresh sweet potatoes, but the number of animals used was too small for reliable interpretation of the bioassay results.

No account is given in any of these papers of the technics employed to assure that the sample of the vegetable analyzed chemically was as representative as possible of the small portions received by the rats as supplements. Discrepancies between chemical analyses and bioassays have frequently been attributed to sampling errors (Bacharach, '41). Kemmerer and Fraps ('45) chromatographed the plant pigments and calculated their β-carotene equivalent, so that the second inaccuracy commonly encountered in measurements of carotene content, i.e., inadequate differentiation among the carotenoids, was eliminated as a source of error in their studies.

Orent-Keiles, Callison, Schaevitz and Frenchman ('46) have published results of experiments comparing the chemical and biological assay of carotene in cooked kale and also com-

paring the carotene so measured with the carotene after extraction from the kale and concentration in cottonseed oil. Particular care was observed in sampling, so that the material analyzed by the two methods was as nearly identical as possible; also the β-carotene of the vegetable was separated from the biologically inactive carotenoids by chromatography. It was found that the vitamin A value of the vegetable accounted for only 67% of the β-carotene as measured chemically. On the other hand, the carotenes extracted from the kale and fed in cottonseed oil solution gave the same vitamin A potency as did an equivalent amount of standard crystalline β-carotene fed in a like manner. This evidence suggested that "incomplete digestion of the kale, and therefore incomplete absorption of the vegetable carotene in the intestinal tract of the animal, may be an important factor causing the difference between the vitamin A values of kale obtained by chemical and biological methods." Callison and Orent-Keiles ('47) found that very large amounts of carotene in the form of cooked carrots were necessary to cure experimental human night blindness produced by a vitamin A-deficient diet. However, when the bioassay vitamin A value of the carrots was used as a basis for calculation of the human requirement, the figures conformed closely with the vitamin A requirement measured by feeding carotene in oil, frozen peas and frozen spinach (Booher, Callison and Hewston, '39; Booher and Callison, '39). These results demonstrated that neither the rat nor the human adult uses carotene from carrots as effectively as that from some other sources.

Such experiments have important implications for the development of tables of food composition to be used for the calculation of human dietary intakes and needs. Therefore, experiments have been carried out to relate quantitatively the vitamin A value and carotene content of the only two carotene-bearing root vegetables in common use, namely, carrots and sweet potatoes, and to compare them with green leafy kale.

In addition, the question has been investigated of whether or not increased fineness of subdivision of the vegetable would

facilitate absorption of the carotene and hence result in a higher bioassay value.

EXPERIMENTAL

A single lot of California-grown carrots identified as the Imperator variety[1] bought on the Washington, D. C., market, was used in these studies. The vegetables were washed in tap H_2O, rinsed in distilled H_2O, pared thinly with a vegetable peeler and cut crosswise into ¼-inch slices. The slices were then cooked by boiling for three minutes in distilled H_2O, by a standardized procedure, and cooled in iced H_2O; small units of the cooked slices were wrapped in wax paper, packed in cartons, quick-frozen and stored at 0°C. until used. The carrots to be fed as puree were comminuted before use by passing them through a food grinder 10 times before thawing.

Similarly, one lot of market sweet potatoes of the Porto Rico variety was studied. This variety was used because of its very high carotene content (Hansen, '45; Ezell and Wilcox, '46). The potatoes were washed thoroughly in tap H_2O, and rinsed in distilled H_2O, steamed in the skins until done and transferred to the refrigerator until cool. The skins were then removed and the sweet potatoes cut into slices. Half of the slices, selected at random, were wrapped in cellophane, packaged in cartons, quick-frozen and stored at —40°C. It was necessary to store the sweet potatoes at a temperature lower than that employed for carrots in order to maintain them in the frozen state. The remaining slices were whipped to a very smooth consistency in a mechanical mixer; approximately 30-gm portions of the "mashed" sweet potato were wrapped in cellophane, packaged, quick-frozen and stored at —40°C.

The difficulties of obtaining truly representative samples for bioassay and chemical analysis from root vegetables with their uneven though symmetrical distribution of carotene were met by following the sampling procedure for carrots out-

[1] The authors are indebted to the Division of Fruit and Vegetable Crops and Diseases, Bureau of Plant Industry, Soils, and Agricultural Engineering, U. S. Department of Agriculture, for identification of the vegetable varieties.

lined by Callison and Orent-Keiles ('47). A wedge-shaped section was cut from the vegetable slice and used for bioassay; the remainder of the slice was preserved for chemical analysis by storing at 0°C.

Biological assay

In conducting the bioassays the criteria for depletion symptoms and length of depletion time recommended in the U. S. Pharmacopoeia (12th ed.) were employed. Weanling rats 21–28 days of age and weighing 40–49 gm were depleted of vitamin A stores on a diet consisting of hot alcohol-extracted casein 18%, cottonseed oil 10%, Osborne and Mendel salt mixture 4%, dried yeast 15%, cornstarch 53%, and viosterol to supply 3 U.S.P. units vitamin D per gram diet. Males and females were used in approximately equal numbers. When the animals declined or remained stationary in weight and displayed eye symptoms, supplementation was begun. Members of litter-mate triads received thrice weekly (a) the β-carotene standard at a level of 8.4 μg per week, (b) the vegetable at a level calculated to supply somewhat less than this amount of vitamin A value, and (c) twice this amount, respectively. Animals were weighed weekly during the 4-week assay period. To calculate the vitamin A potency of the vegetable, average growth rates of the two groups of animals that received the vegetable were related to the growth rate of the comparable group receiving the standard. When one animal of a triad failed to complete the assay period, data from the other two animals was also discarded, so that the average growth rates reported represent strict litter-mate comparisons. The sweet potatoes, both sliced and mashed, actually were fed at 4 levels because growth rates for the first week indicated that the original levels chosen were perhaps too low. However, since growth rates on the 4 levels resulted in vitamin A values which were in good agreement with one another, all the data were included in the final evaluation.

In the assays of the vegetable carotene extracts, litter-mate pairs of rats received the standard β-carotene and the amount

of extract calculated to contain the equivalent of 8.4 μg β-carotene per week, respectively. Each pair was isogenic with a triad used in the assay of either the sliced or pureed form of the vegetable.

Chemical analysis

At approximately 10-day intervals during the progress of the bioassays the carrots which had been reserved for chemical analysis were thawed, each slice divided in half, and a 12.5-gm aliquot of each lot of pooled halves taken for duplicate analyses. The sample was agitated for 5 minutes in the Waring Blendor with a 1:1 mixture of acetone-absolute methanol and then transferred quantitatively to a beaker and allowed to extract 24 hours longer at 0°C. It was then filtered by suction and washed with small portions of acetone-methanol, followed by three washings with petroleum ether (Skelly-solve, fraction F, purified and distilled, b.p. 40°–60°C.). The carotenols were removed by treatment with saturated methanolic KOH and sufficient distilled H_2O was added to give good phasic separation. Traces of pigment which remained in the residue were extracted by repeated alternate washings with small portions of acetone-methanol and petroleum ether. The carotenoids were then completely extracted from the acetone-methanol layer with petroleum ether and the combined extracts washed free of traces of carotenols with methanolic KOH. Finally the petroleum ether extract was washed with distilled H_2O until neutral, the excess water removed by "freezing out" at a low temperature, and the extract diluted to volume and stored over anhydrous Na_2SO_4 until used. Aliquots of this extract were taken for: (1) measurement of total carotene content; (2) chromatographing; and (3) dilution with cottonseed oil for bioassay of the carrot extract. The total carotene content of the unchromatographed extract was determined in terms of β-carotene by measuring absorption at 450 mμ with the Beckman

spectrophotometer. The chromatographing was done on ice water-cooled columns of a 1:3 MgO,[2] Hy-flo Supercel mixture. The band of α-carotene was eluted with petroleum ether and the β-carotene with 2% ethanol in petroleum ether; the band of biologically inactive carotenoids was cut from the column and eluted from the adsorbent with the ethanol-petroleum ether mixture. The several fractions were diluted to convenient volumes and read at 430, 450, and 480 mμ; calculations of carotene content were based on a standard β-carotene curve obtained with the same spectrophotometer.

Sweet potatoes were similarly sampled. The extraction procedure was carried out essentially according to the method described by O'Connor et al. ('46), except that extraction of pigments from the vegetable was accomplished by agitation in a Waring Blendor with cold 95% ethanol for 10 minutes, followed by standing in contact with 95% ethanol for 16–18 hours at 0°C. The extract was then filtered and the residue re-extracted until all color had been removed. Also petroleum ether was used in place of the iso-octane which O'Connor et al. recommend. Cooled MgO Hy-flo Supercel columns were employed in chromatographing, and the β-carotene and non-active pigments removed in separate fractions with 2% ethanol-petroleum ether. Pigments in the final solutions were measured in the same way as in the case of carrots.

RESULTS

The total yellow pigment content of the Imperator carrot slices analyzed was equivalent to 102 μg β-carotene per gram cooked carrot, with a standard error of 9.0; the puree samples averaged 104.0 ± 9.8 μg per gram. Of these total pigments 63.2% and 62.2%, respectively, was β-carotene, 25.3% and 25.5% α-carotene, and the remainder consisted of biologically inactive carotenoids. Recoveries from the chromatographing columns averaged 90% of the total pigments. The amount of β-carotene present in this sample of Imperator carrots was somewhat higher than the 51 μg per gram re-

[2] Magnesium oxide powder, "Baker's Analyzed" reagent.

ported by Harper and Zscheile ('45) for the same variety, and the per cent of α-carotene found was considerably lower than the average of 46% for the "garden varieties" studied by the same authors.

The total yellow pigments of the sliced or mashed Porto Rico sweet potatoes averaged, in terms of β-carotene, 57.4 ± 7.4 and 54.1 ± 4.1 μg per gram vegetable, respectively. β-carotene was the only biologically active carotenoid present and constituted 81.8% of the yellow pigment in the sliced vegetable and 79.2% in the mashed vegetable. Recoveries were similar to those obtained with carrots. This ratio of β-carotene to total yellow pigment agrees with the average figure of 81.8% reported by Ezell and Wilcox ('46). However, both the total yellow pigment and the β-carotene content of the sweet potatoes analyzed in this study were in the higher range of the values reported by these authors.

The presence of the isomers of β-carotene, neo-β-carotenes B and U (called neo-β-carotene and carotenoid X by Kemmerer and Fraps) have been demonstrated in certain fresh vegetables (Beadle and Zscheile, '42; Kemmerer and Fraps, '45). These pigments are inseparable from β-carotene when adsorbed on MgO and therefore are included with the β-carotene fraction in this study.

Since neo-β-carotene B (Kemmerer and Fraps, '43) has been shown to possess about one-half and neo-β-carotene U (Kemmerer and Fraps, '45) only one-quarter to one-third the vitamin A activity of β-carotene, their presence in the β-carotene fraction in any considerable amounts would significantly alter the potential vitamin A value. Kemmerer and Fraps ('45) found no evidence of either isomer in fresh carrots and only slight traces of the B isomer in dehydrated carrots. Sweet potatoes they showed to contain carotenoid X (neo-β-carotene U) and neo-β-carotene (neo-β-carotene B) in amounts of 5.4% and 3.7%, respectively, of the total "crude" carotene. No figures for kale are available. The ratios of the absorption at 430 mμ and at 480 mμ to that at 450 mμ indicate the possible presence of isomers in the β-carotene fraction

from all three vegetables in the present study. However, since the unchromatographed extracts of carotenoid pigments from the vegetables studied gave bioassay values which agreed well with the vitamin A potency calculated from chemical analyses, it is unlikely that these two less active isomers were present in significant amounts.

Table 1 contains a summary of the growth rates of the animals receiving the several supplements, together with their standard errors. Comparison of the biological and chemical data (table 2) indicates that the proportions of biologically active carotenoids available to the rat for suboptimum growth (bioassay) are very similar for the two yellow root vegetables studied and amount to 34–41% of their potential vitamin A value, which is considerably less than the 67% availability obtained with kale (Orent-Keiles et al., '46).

There were no significant differences among the three vegetables when the carotene was extracted and fed suspended in oil; under these circumstances availability was practically complete. Fine subdivision of the carrots fed to the assay animals increased availability by only 7%; a similar procedure applied to the sweet potatoes left the availability unchanged.

Since these data were obtained, a study by Booth ('47) has been published in which the availability of carotene was investigated with 7 varieties of English carrots; chemical measurements, either with or without chromatographic separation of pigments, were compared with biological values measured either by standard rat growth technics or a liver storage procedure. Studies of fecal excretion of carotene were included. This investigator reported the vitamin A values, calculated from chemical measurements, to range from 4,900 to 93,000 international units per 100 gm wet tissue, which may be compared with a similarly derived average figure of 13,000 international units for the Imperator variety of carrots used in the present experiments. The biological values for the carrots in the English study ranged from 2,900 to 37,000 international units per 100 gm wet tissue; the percentage availability varied from 24 to 70 and tended to be less as the concentration of

TABLE 1

Summary of bioassay data used in calculating vitamin A value of carrots, sweet potatoes and kale

SUPPLEMENTS	NO. OF ANIMAL TRIADS	AVERAGE WEIGHT GAINS, ± STANDARD ERROR, OF ANIMALS RECEIVING THE FOLLOWING SUPPLEMENTS: Vegetable "Low" level	Vegetable "High" level	Carotene extract	β carotene standard 8.4 μg/week	VITAMIN A VALUE [1]
		gm/week	gm/week	gm/week	gm/week	IU/gm
Carrots, cooked:						
Sliced, 0.15 and 0.30 gm/week	33	9.4 ± 3.02	15.7 ± 3.28		16.2 ± 2.70	45
Pureed, 0.15 and 0.30 gm/week	28	10.6 ± 3.37	18.0 ± 2.85		16.6 ± 2.70	52
Extract, ≡ 8.4 μg carotene/week	25 [2]			15.5 ± 3.67	16.3 ± 2.78	
Sweet potatoes, cooked:						
Sliced, 0.24 and 0.48 gm/week	16	7.9 ± 3.36	14.8 ± 3.55		14.4 ± 2.89	..
Mashed, 0.24 and 0.48 gm/week	29	10.3 ± 3.14	14.6 ± 2.99		16.1 ± 3.12	
Sliced, 0.30 and 0.60 gm/week	22	10.8 ± 3.23	16.6 ± 3.46		15.0 ± 3.46	29
Mashed, 0.30 and 0.60 gm/week	24	10.1 ± 2.46	17.2 ± 3.55		15.3 ± 4.01	26
Extract, ≡ 8.4 μg carotene/week	32 [2]			15.9 ± 3.35	15.7 ± 3.78	..
Kale, cooked:						
0.09 and 0.18 gm/week	19	7.2 ± 3.21	12.6 ± 3.00		15.8 ± 3.51	60
Extract, ≡ 8.4 μg carotene/week	20 [2]			12.4 ± 3.29	13.4 ± 2.62	..

[1] Vitamin A value was calculated by plotting growth response against vegetable intake and determining the amount of vegetable equivalent to the carotene standard from this curve. In the case of the sliced carrots and kale, the growth response to the standard was somewhat greater than the response to the "high" level of vegetable; in these cases the curve was extrapolated.

[2] Litter-mate pairs instead of triads were used in the assay of the extract.

TABLE 2

Comparison of vitamin A value of carrots, sweet potatoes and kale, as determined from carotene analysis and rat-growth bioassay [1]

VEGETABLE	NO. OF CHEMICAL ANALYSES	NO. TRIADS OF ANIMALS IN BIOASSAY	CAROTENE CONTENT [2]		VITAMIN A VALUE		$\frac{\text{VITAMIN A (BIOL. IU)}}{\text{VITAMIN A (CHEM. IU)}^{4}} \times 100$
			α	β	Calculated from carotene [3]	Bio-assay	
			$\mu g/gm$		IU/gm		%
Carrots, cooked:							
Sliced	46	33	26	66	132	45	34
Pureed	10	25	25	64	127	52	41
Extract	10	25					96
Sweet potatoes, cooked:							
Sliced	22	45	0	47	78	29	37
Mashed	22	46	0	43	71	26	37
Extract	10	32					101
Kale, cooked [5]	64	19	0	54	90	60	67
Extract	64	20					93

[1] β-Carotene in cottonseed oil used as reference standard.

[2] All measurements based on wet weight of vegetable.

[3] Six-tenths μg *β-carotene and* 1.2 μg α-carotene equated to one international unit vitamin A.

[4] Booth ('47) has used the term "chemical IU" to distinguish between international units calculated from chemical measurements and those determined directly by bioassay. The figures in this column would therefore represent the per cent to be used in changing chemical IU to biological IU.

[5] Orent-Keiles, Callison, Schaevitz and Frenchman, '46.

carotene in the carrot increased. However, for "ordinary" carrots this percentage was 46, which is only slightly higher than the value obtained in the present study.

The results of Booth's experiments, as well as those reported here, lend support to the idea that by an accumulation of data relating chemical and biological vitamin A values obtained with well-controlled sampling and analytical procedures, it may be possible to obtain a suitable correction figure for each vegetable which can be used to reduce chemically and biologically determined vitamin A values to a common basis for use in dietary calculations. Further, the ratio of biological vitamin A values to those calculated from chemical measurements of carotene content proved to be very similar for the two yellow root vegetables studied, and only about half of that obtained from a green leafy vegetable (kale). This suggests that it may be possible to derive a single conversion factor for each general class of carotene-containing vegetables and fruits.

Additional experimentation with human subjects is necessary to determine the relative availability to the human being of carotene from various sources. The somewhat scanty information available at present indicates that whether or not the human being utilizes carotene as completely as does the rat depends upon the nature of the vitamin A active material fed (Booher, Callison and Hewston, '39; Booher and Callison, '39; Callison and Orent-Keiles, '47).

SUMMARY

Chemical measurements of the biologically active carotenoids of the two principal yellow root vegetables, carrots and sweet potatoes, were compared with the biological vitamin A value obtained by rat growth bioassay of as nearly identical a sample of the vegetable as possible. In addition, study was made of the effect upon the availability of carotene to the animal of comminution of the vegetable and of extraction of carotene from the vegetable matrix. The carrots were of the

Imperator and the sweet potatoes of the Porto Rico variety, and both were cooked before analysis.

The carrots averaged 26 µg of α-carotene and 65 µg of β-carotene per gram of cooked vegetable; the biological vitamin A value was 34% of that calculated from the carotene content when the carrots fed were sliced, and 41% when they were pureed. The sweet potatoes averaged 44 µg of β-carotene per gram cooked vegetable and bioassay gave 37% of this value regardless of whether the sweet potato was sliced or mashed. The percentage availability of carotene from these yellow root vegetables is contrasted with 67% for cooked kale, reported earlier from this laboratory.

The feasibility is indicated of obtaining a factor for each vegetable, or possibly each class of carotene-bearing plant foods (yellow or red fruit, yellow root, green leafy, green leguminous vegetables), which could be used to convert provitamin A content in terms of carotene to the equivalent international vitamin A units, derived biologically, or vice versa. A conversion of this nature is necessary in order either to combine or to compare with any degree of validity vitamin A values obtained by the two methods.

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EFFECT OF ENVIRONMENT ON GROWTH AND FEED AND WATER CONSUMPTION OF CHICKENS

II. THE EFFECT OF TEMPERATURE AND HUMIDITY OF ENVIRONMENT DURING THE FIRST EIGHTEEN DAYS AFTER HATCH

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TWO FIGURES

(Received for publication July 23, 1948)

INTRODUCTION

In the first paper of this series (Barott and Pringle, '47) it was shown that with chicks brooded from hatch to 9 days of age maximum growth and efficiency of feed utilization resulted when the temperature was 94°–95°F. the first day, dropping uniformly to 88°F. on the 9th day. This research has been extended in the present experiment to cover the first 18 days after hatch and to include the effect of humidity.

PROCEDURE[2]

The work was continued in the small houses with environmental control described in the earlier paper. The same

[1] Acknowledgment is made of the assistance of James Jackson, who aided in the general routine of conducting these experiments.

[2] One important detail should have been mentioned in the original paper. As stated, all chick cages were equipped with wire mesh floors. As made by the manufacturers these floors are from 1½" to 2" below the top of the feed trough. This is not conducive to prompt feeding of day old chicks. Therefore, in all experiments currently reported this floor was raised to the level of the feed troughs for the first few days and thereafter gradually lowered, reaching the original position at about the 10th to 12th day after hatch.

equipment, feed formulas and controls were used and the routine followed was identical. The chicks, Rhode Island Reds, were obtained from the same flock and 30 chicks were used in each experiment as before. Mortality was practically nil.

As was explained in the first paper, all chicken weights were corrected for the amount of extraneous matter in the system. To determine this amount, 30 chickens were dissected daily from the 9th to 18th day of age. The contents of the gizzard and intestinal tract were removed and weighed separately and an average weight for the 30 chickens obtained. (All weights of chickens used in these experiments were obtained 4 hours after the last feeding period; consequently there was no feed in the crops, and normally all yolk had been utilized by the 7th day.)

Values obtained for undigested food material in the system increased lineally from 7.20 gm on the 9th day to 11.30 gm on the 18th day.

TABLE 1

Temperatures on the 18th day after hatch for the 53 experiments

Number of experiments	3	3	10	11	10	3	5	4	4
Temperature on 18th day (F°)	86	83	82	81	80	79	78	74	70

RESULTS AND DISCUSSION

Effect of temperature

The temperature for the first 9 days after hatch was that reported as the optimum in the first paper: i.e., 94°–95°F. at the start, dropping uniformly to 87°–88°F. on the 9th day.

To determine the optimum temperature from the 9th to the 18th days, 53 experiments were conducted with a total of 1590 chickens. The temperatures on the 18th day were as shown in table 1.

On the basis of comparative analyses of the results obtained for all experiments conducted within a given temperature range, the gain in weight of the chickens between the 9th and 18th days in that range of temperature was computed as a

per cent of the weight on the 9th day. The gain was also computed for each lot of chickens for 18 days (from the start to the 18th day) as a per cent of the initial weight. The results so obtained for each temperature range were plotted, as in figure 1, and curves drawn through the plotted points.

The numeral at each point designates the number of experiments made to obtain that point: e.g., a point with the figure 10 indicates that 10 experiments of 30 chicks each, or a total of 300 chickens, were used to obtain a mean value for that point.

The point is plotted at the mean temperature prevailing during the period investigated for the particular group.

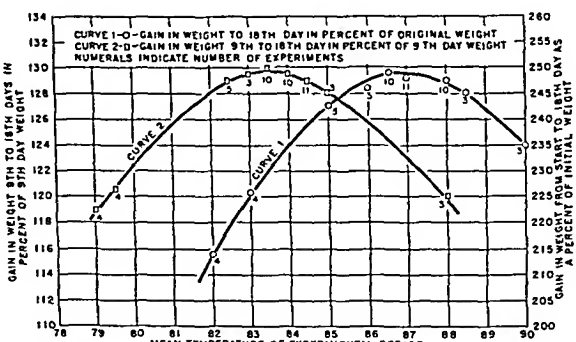

Fig. 1 Effect of temperature of environment on growth.

Curve 2, which represents growth between the 9th and the 18th days, shows a definite variation due to temperature. The maximum growth occurred when the mean temperature was 83.5°F. (87°F. on the 9th day and 80°F. on the 18th day) and resulted in a 130% increase in the last 9 days over the weight on the 9th day. The gain in weight was less at other temperatures and became less and less the further the temperature deviated above or below that found to be associated with maximum growth.

Curve 1, which gives the growth in 18 days as a per cent of the initial weight, shows that the maximum growth occurred at a mean temperature of 87°F. (94°F. at the start and 80°F.

Curve 5 shows the efficiency of feed utilization: i.e., gain in weight per gram of feed eaten. The values from which the curve was plotted were obtained, as in the previous paper, by dividing values taken from curve 1 (daily gain in weight) by values taken from curve 2 (daily feed eaten, in grams) at the corresponding time.

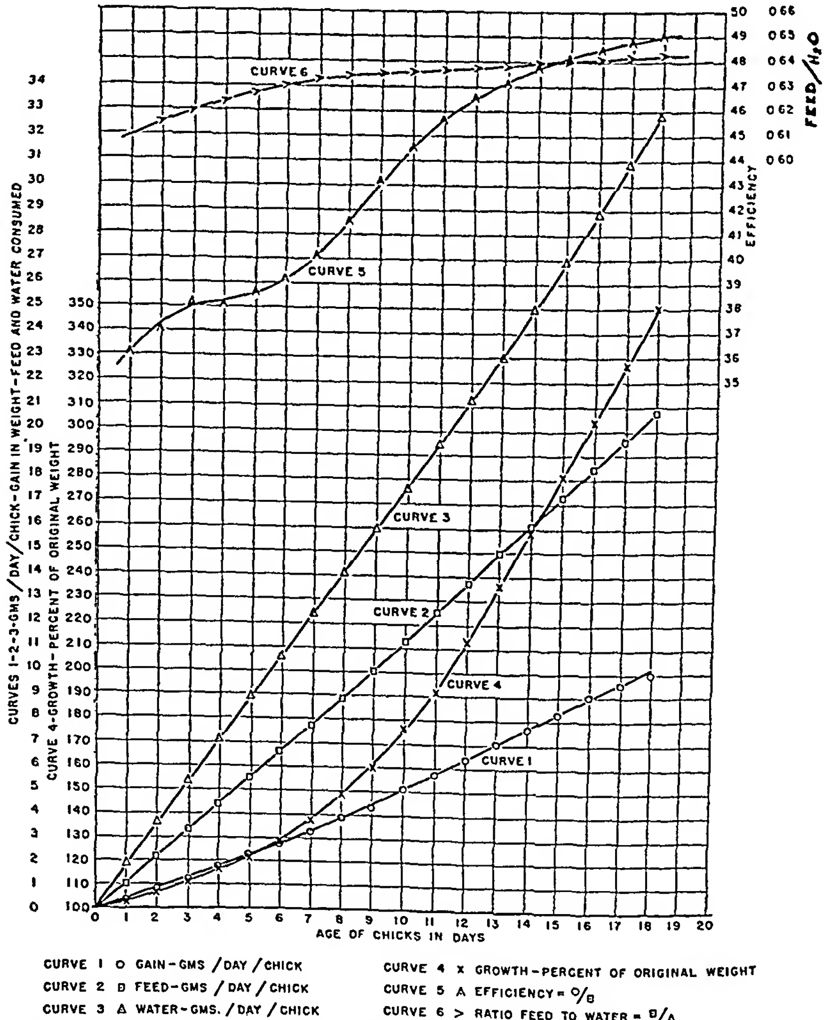

Fig. 2 Growth and feed and water consumption of chickens from hatch to 18 days of age.

The efficiency rose from a value of 0.43 on the 9th day to 0.49 on the 18th day. The increase in efficiency is most rapid between the 6th and the 10th days, and gets less and less each day, from the 10th to the 18th. Between the 7th and 9th days it rose from 0.40 to 0.43, while from the 17th to the 18th it rose from 0.485 to 0.490.

The figure of 0.49 for efficiency of feed utilization on the 18th day (curve 5) is higher than that found in the results published by other investigators. We feel that this higher value is more correct because of the unusual care taken in the present work to measure accurately the amount of feed eaten.

The change in weight of the troughs from day to day does not indicate the amount of feed eaten as accurately as might be supposed. There is a change in weight due to moisture gained or lost from the feed, depending upon whether the hover is at a higher or lower humidity than the place of feed storage. But the greatest correction to the loss in weight of the feed trough from day to day is due to the feed which is thrown from the trough by the chickens. If the conditions of the experiment are such that this waste feed cannot be accurately determined, then all values for feed eaten are too great and all values for efficiency too low.

The correction for change in weight of the feed troughs due to moisture gained or lost to the atmosphere was determined by exposing a trough, identical with the feed troughs and containing the same amount of feed, to the atmosphere of the brooder room but out of the reach of the chickens. The change in weight of this trough was obtained each day and applied as a correction factor to the food eaten.

The correction so obtained may be too small because the surface of the feed in the check trough was undisturbed, while that in the feed troughs was continuously changing due to the chickens feeding. However, this correction is not large, amounting to from one to 4 gm per day. During the first few days, when the chickens are small and not eating so much as later, this amount is important, as a three-gram

change in weight amounts to about 3% or more of the feed eaten. This correction, as a per cent of total feed eaten, becomes less and less important as the chickens grow older and the consumption of feed increases.

The loss due to feed that was thrown from the trough by the chickens was determined by carefully collecting the waste feed and weighing it. It is important that this correction be determined accurately, because the amount of feed thrown from the trough may in extreme cases equal as much as 50% of the feed eaten.

TABLE 3

The effect of humidity on growth

RELATIVE HUMIDITY	NUMBER OF EXPERIMENTS	INCREASE IN WEIGHT		
		0 to 9th day as a per cent of initial weight	0 to 18th day as a per cent of initial weight	9th to 18th day as a per cent of weight on 9th day
%		%	%	%
35	3	48	134	245
50	3	52	130	244
55	8	50	133	240
60	9	52	126	240
65	10	52	128	245
70	1	50	126	236
75	3	51	127	242

Effect of humidity

A total of 37 experiments (1110 chicks) were conducted with relative humidities ranging from 35% to 75%. Little, if any, difference in growth was noted, as can be seen by the results shown in table 3. The temperature in these experiments ranged from 93°–95°F. at the start to 78°–83°F. on the 18th day.

SUMMARY

Chickens were housed in a controlled environment without a hover for the first 18 days after hatch, to determine the optimum temperature for growth and efficiency of feed utilization. Thirty chickens were used for each experiment and a total of 53 experiments were performed. Equipment, feed formulas and routine of procedure were the same as for

work reported in a pevious paper by the present authors ('47).

The temperature during the first 9 days was kept at the optimum as reported in the first paper: i.e., 94°–95°F., at the start, and then dropped uniformly to 87°–88°F. on the 9th day. For the period from the 9th to the 18th days the temperature was varied with different lots of chickens from a temperature of 87°–88°F. on the 9th day to temperatures ranging from 86°–70°F. on the 18th day.

Maximum growth over the period from the 9th to the 18th day was found when the temperature dropped uniformly from 87°F. on the 9th day to 80°F. on the 18th day. The growth under these conditions equaled a 130% increase in weight over the weight on the 9th day.

The efficiency of feed utilization rose from a value of 0.43 on the 9th day to 0.49 on the 18th day. Both growth and efficiency became less as the temperature was varied either way from the range noted for maximum growth, and the greater the variation the greater the difference.

The amount of feed consumed increased approximately 1 gm per day per chick. On the 18th day after hatch the chicks consumed approximately 2.02 gm of feed for each gram gain in weight and drank approximately 1.55 gm of water for each gram of feed eaten.

Humidity seems to make little if any difference in growth within the range here reported.

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THE PROTEIN REQUIREMENTS OF COLLEGE WOMEN ON HIGH CEREAL DIETS WITH OBSERVATIONS ON THE ADEQUACY OF SHORT BALANCE PERIODS [1, 2]

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THREE FIGURES

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Nitrogen balance studies have been used routinely in assessing the protein requirements of adults. The criterion of adequacy has been the establishment of nitrogen equilibrium. In conducting these studies, short periods have generally been used. Sherman ('20) reported the protein requirement of one subject consuming bread protein for 5 consecutive three-day tests, as well as data for several other subjects on diets for an indeterminate number of days. Murlin et al. ('46a, '46b) in a series of studies on nitrogen metabolism, routinely employed three- to 5-day periods in comparing the value of various nitrogen constituents for maintaining nitrogen equilibrium in man. Hegsted et al. ('46) studied the protein requirements of adults using periods of 6 days or longer. Bricker, Mitchell and Kinsman ('45) in testing protein requirements used 5 to 12 days, depending on uniformity of balances. The present

[1] A preliminary report of these data was presented before the American Institute of Nutrition in Atlantic City, March, 1948.

[2] Aided by a grant from General Mills, Inc.

[3] Now located at the School of Home Economics, Florida State University, Tallahassee.

investigation was undertaken to estimate the validity of short time studies in predicting the protein requirement of adults for a longer period, i.e., 10 weeks.

EXPERIMENTAL PROCEDURE

General plan

The individual protein requirements of 9 college women consuming a diet in which cereals supplied 70% of the protein were estimated in the initial phases of this study by short balance periods. The efficacy of the diet for maintaining the subjects in good health was tested in a subsequent 10-week period, during which 10 subjects (the original nine, plus an additional subject) were fed according to the protein requirements assessed for them. Constancy of nitrogen balance data, body weight, hematological data and performance tests were used to assess adequacy of dietary protein.

Subjects

Ten college women (9 for the entire study and one additional during the last 70 days), 19 to 30 years of age, selected on the basis of health and willingness to cooperate, served as subjects. Health status was determined by examinations by the University Health Service medical officers. The initial age, height, weight and surface area of the individual subjects are shown in table 3.

Dietary regime

The diets were composed of a basal diet low in nitrogen plus the protein mixtures being tested. The low nitrogen foods and the quantities consumed by one of the subjects are listed in table 1. Sugar and butterfat, in proportions to supply one-third of the calories as fat, were used to adjust individual caloric needs. Maintenance of body weight was used as the criterion of caloric adequacy. In general, 45 cal. of food per kilogram of body weight per day sufficed for weight maintenance at all levels of nitrogen intake. Subjects D, F, and H

TABLE 1

Sample daily basal diet of a 48 kg subject together with calculated[1] values for fat and caloric content

COMPONENT	AMOUNT	ENERGY VALUE	FAT
	gm	*Cal.*	*gm*
Cornstarch cookies:			
Cornstarch	196.5	692	.
Sugar	19.5	78	
Butterfat[2]	37.5	338	37.5
Corn oil	3.8	34	3.8
Agar, powdered	3.0		
Salt mixture[3]	16.4		.
Lemon juice	100.0	33	.
Sugar	158.0	632	
Butterfat[2]	39.0	351	39.0
Total		2158	80.3
Applesauce[4]	100.0	80	.
Lettuce[4]	30.0	5	
French dressing[4]	15.0	90	10.0
Total		175	10.0
Vitamin supplement[5]			.

[1] Bradley ('42).

[2] Washed free of salt and curd.

[3] For formula see footnote 4, bottom of page.

[4] Omitted in period 3.

[5] Supplied by two Stuart Formula Tablets daily (Stuart Laboratories, Pasadena, California). These furnished the following: thiamine chloride, 4.0 mg; riboflavin, 4.0 mg; nicotinamide, 30.0 mg; calcium pantothenate, 5.0 mg; pyridoxine, 0.2 mg; ascorbic acid, 100.0 mg; mixed tocopherols, 4.0 mg; vitamin A, 5000 USP units; vitamin D, 800 USP units.

were exceptions, the first two requiring only 35 cal. and the latter 50 cal. per kilogram.

The salt mixture[4] incorporated into the cookies was devised to supply the mineral needs of the subjects and to

[4] This salt mixture was made according to a formula supplied by Dr. W. C. Rose, Department of Biochemistry. It contained 5.6 gm baking powder, 3.75 gm mineral salts, and 7.1 gm NaCl. The baking powder, made in this laboratory, contained 27.0% $NaHCO_3$, 37.6% $Ca(H_2PO_4)_2.H_2O$, and 35.4% cornstarch. The mineral salts in the proportions used were: $CaCO_3$, 32.14; $KHCO_3$, 50.18; $MgSO_4 \cdot 7H_2O$, 13.78; $FeC_6H_5O_7 \cdot 3H_2O$, 3.72; $MnSO_4 \cdot 4H_2O$, 0.0472; $CuSO_4 \cdot 5H_2O$, 0.1670; and KI, 0.0094.

serve as a leavening agent for the cookies. The high levels of riboflavin and nicotinamide were given as a precaution against the possibility that lowered protein intakes might increase riboflavin requirements (Oldham and co-workers, '47) or that lowered tryptophan intakes incident to low protein intakes might increase niacin requirements (Krehl et al., '45).

The protein mixture chosen for study (see table 2) was one devised in connection with another study on the relative protein value of diets in which 30 or 70% of the protein was of cereal origin. The 70% cereal protein diet, as the latter diet will be termed hereafter, was selected for this long term study

TABLE 2

Quantities fed and protein contribution of protein foods used in estimation of individual nitrogen requirements

SOURCE OF PROTEIN	WEIGHT OF FOOD		PROTEIN CONTRIBUTED
	Period 1	Period 2	
	gm	gm	%
Cereal foods			
White bread, water	61.5	123.0	59.5
Oats, rolled	7.0	14.0	10.5
Non-cereal foods			
Beef, lean ground	7.4	14.8	14.7
Cream, 20%	43.4	86.8	13.4
Potatoes, white	9.0	18.0	1.9

because its use would allow the inclusion of a larger volume of natural foods than would a protein mixture composed predominantly of proteins of animal origin. This inclusion of natural foods was deemed desirable to avoid monotony in an otherwise uninteresting, purified diet. The reasoning behind the choice of types and proportions of proteins is set forth in a paper dealing with a comparison of the 70% and 30% cereal proteins (Bricker and Smith, unpublished). The composition of the protein mixture and the per cent protein contributed by the individual foods are shown in table 2. The percentage of total protein supplied by each of the foods was as follows: white bread, 59.5; oatmeal, 10.5; beef, 14.7;

cream, 13.4; and potato, 1.9. When these foods were added to the basal diet, adjustments were made so that fat continued to supply one-third of the total calories (which were also adjusted to supply 45 cal./kg body weight).

TABLE 3

Age, body size and basal metabolism of 10 women subjects on a study of protein requirements

SUBJECT	AGE	BODY WEIGHT	STANDING HEIGHT	SURFACE AREA [1]	BASAL METABOLISM [2]
	years	*kg*	*cm*	*m²*	*cal./24 hr.*
A	24	46	164.5	1.48	1257
B	19	67	170.8	1.78	1486
C	24	55	157.5	1.55	1197
D	30	58	167.0	1.65	1245
F	25	76	180.3	1.96	1469
G	22	48	156.8	1.45	1010
H	22	50	162.6	1.52	1200
I	24	66	167.0	1.74	1249
J	26	70	165.7	1.78	1248
K	22	65	173.4	1.78	1343

[1] Estimated by the DuBois ('16) formula, $\mathrm{Wt.}_{\mathrm{kg}}^{0.425} \times \mathrm{Ht.}_{\mathrm{cm}}^{0.725} \times 71.84$.

[2] Determined by oxygen consumption measured by the McKesson Waterless Metabolor.

The lengths of the three initial feeding periods described below were those shown by previous experience to allow sufficient adjustment to the new dietary regime.

Order of feeding

Four test periods, 10 to 70 days in length, were used in the order shown below:

Period 1 — 70% cereal protein diet supplying 1.6 gm nitrogen, 10 days.

Period 2 — 70% cereal protein diet supplying 3.2 gm nitrogen, 10 days.

Period 3 — Low protein diet supplying 0.2 gm nitrogen, 15 days.

Period 4 — 70% cereal protein diet fed according to individual nitrogen requirements, 70 days.

During the first 10-day period, 4 subjects (A, F, G, and J) were placed on the 1.6 gm intake and 5 subjects (B, C, D, H, and I) on the higher level of 3.2 gm of nitrogen. They were fed in reverse order for the next 10 days. The period ended on December 19, prior to the Christmas holiday when subjects were allowed free choice of food. Period 3, extending from January 4 to 19, was followed by a two-day break in which the subjects consumed an ordinary mixed diet supplying 50 gm protein daily. Period 4 extended from February 13 to April 23. This period was postponed in order to allow time for analysis of samples and data necessary for computation of individual protein needs. Two test periods intervened between periods 3 and 4. During this intervening time the subjects received 3.0 gm nitrogen daily from bread or meat, the order of feeding depending on the individual schedule. Subject K started on the study at the beginning of period 3.

Calculation of individual protein requirements

The individual requirements used in constructing the diets fed during the 10-week period were calculated from the data obtained during the first three periods. These requirements were estimated according to the technique described in the first paragraph of the section entitled Results.

Collection and preparation of samples

Foods were divided for analysis into 4 sample groups, as follows: cookies; lemon juice, lettuce, and applesauce; bread and oatmeal; and potatoes, meat, and cream. The samples were pooled in 5-day composites except during period 4, when 7-day pools were made.

Twenty-four hour urine samples were collected and preserved with 1 to 2% acetic acid. Fecal samples were marked with ferric oxide or chromic oxide. Fecal collections were made during the last 5 days of periods 1 and 2, during the last 10 days of period 3, and in weekly intervals throughout period 4.

As soon as foods were sampled and feces collected they were refrigerated. After pooling, the samples were acidified with 50 to 100 ml glacial acetic acid, blended in a Waring Blendor and made up to a given weight with distilled water.

Methods of assay

All nitrogen analyses were made by the Kjeldahl method. Hematologic determinations were made weekly on fasting capillary blood. Hemoglobin was determined colorimetrically by the acid hematin method of Newcomer. Erythrocytes were counted by an accepted technique (Gradwohl, '43). For these counts the blood was diluted with Hayem's solution and an improved Neubauer counting chamber calibrated by the National Bureau of Standards was used.

Performance tests

In this portion of the study tests were used (with the exception of the Rohrschach) which have been reported by one or more authors as being sensitive to dietary deficiency or change. The critical fusion frequency of flicker, speed of tapping, coordination time and manual steadiness in holding a static position were studied according to the techniques listed by Keeton et al. ('46). Physical endurance was measured by use of a recording electrodynamic brake bicycle ergometer (Kelso and Hellebrandt, '34) according to the following procedure: the subject rode against resistance of 6 amperes at the rate required to maintain an output of 4 volts until unable to pedal further. After a 10-minute rest the test was repeated. Graphic recordings of the work output were made by an electric kymograph. The endurance score was expressed as the total number of seconds during which the desired voltage was maintained.

Other tests used in an attempt to determine possible effects of the diet on mental function and personality were proofreading, memory, code learning, reading, and Rohrschach multiple choice tests. The techniques and forms used are described below:

Proofreading. This test was fashioned after Thurstones' "Scattered X'S" (Thurstone and Thurstone, '41), which they classed as a test of the preceptual factor of intelligence. Tests were performed the first, third, 5th, 7th, and 9th weeks of the study.

Memory tests. These tests, given during the weeks 1, 2, 4, 6, 8, and 9, were also made according to a form reported by Thurstone ('37), containing lists of numbers and objects, numbers alone, and names with initials to be memorized. Two modified forms were constructed in which names and numbers of objects were changed.

Code learning. The test form used[5] here consisted of 4 parts, covering number checking, code translation, finger dexterity, and counting. The same form was used throughout and tests were made during the first, third, 5th, 7th, and last weeks of the experiment. Harrell ('43) has reported that thiamine-fed subjects improved significantly more in performance of this test than did controls fed placebos.

Reading tests. The tests chosen here were the advanced Iowa Silent Reading Tests, forms Am, Bm, Cm, and Dm.[6] The parts measuring rate and comprehension were used in tests during the first, 4th, 7th, and 10th weeks. One form was used in each of the 4 test in the order listed above.

Rohrschach. This test, given during the first, 6th, and 10th weeks, was made by using "Harrower-Erickson Multiple Choice Test" blanks (Harrower-Erickson and Steiner, '45) with the Rohrschach cards.

It was deemed wise to gauge the extent of a learning factor by conducting the above performance tests for the same length of time on a control group of subjects on freely chosen diets. Nine subjects ranging in age from 20 to 26 years, seniors in nutrition, were selected on the basis of interest in the study and willingness to cooperate. These subjects were not fed weighed diets but selected their diets according to the

[5] Test 2, Code Translation from the "Survey of Working Speed and Accuracy" constructed and standardized by Floyd Ruch, California Test Bureau, 1943.

[6] Iowa Silent Reading Tests, new edition published 1943 by the World Book Company, Chicago, Illinois.

pattern recommended by the Food and Nutrition Board of the National Research Council.[7] Good cooperation in achieving this pattern was evidenced by special efforts made to secure foods not provided by their regular diets. These girls will be referred to in the discussion as control subjects and those on weighed diets as protein subjects.

RESULTS OF THE INVESTIGATIONS

The nitrogen balance data of periods 1, 2, and 3 plotted against the nitrogen intakes for 9 of the subjects are shown in figure 1. Subject K did not participate in this part of the study. Data at the 3.2 gm intake are missing on the graphs for subjects F and J since they were ill with influenza at the time. Regression lines have been fitted to these points by the method of least squares. The resulting regression equations shown on the individual graphs have been used to assess the amount of food nitrogen needed for equilibrium as well as the amount required for "adult growth" (Bricker et al., '45). The net amount of nitrogen needed for "adult growth" was estimated by allowing 0.77 gm nitrogen per square meter of surface area. The gross amount of nitrogen was obtained by substituting these net values as Y in the individual regression equations shown for each subject on figure 1. The values thus obtained represent the total amount of dietary nitrogen required for equilibrium and "adult growth." These values for each subject are presented in table 4. Translated in terms of protein ($N \times 6.25$), the amounts required (all subjects except J) in grams were 31.7, 35.2, 27.1, 30.2, 30.9, 26.9, 29.4, 42.2, and 32.0, with an average of 31.7 gm per day. The value for J was not included in the average since it was shown later to be an underestimate.

The average daily nitrogen balances and body weights of the subjects for each week of the 10-week period are shown in figure 2 and the average for all subjects (except J) in figure 3. The balances remained positive throughout the

[7] Circular no. 122, Food and Nutrition Board, National Research Council, August, 1945, p. 16, list I.

10 weeks for all subjects except J and D, with no tendency for a less positive balance at the end of the period. It will be seen that the ingestion of the quantity of protein estimated to be needed by subject J resulted in values around equili-

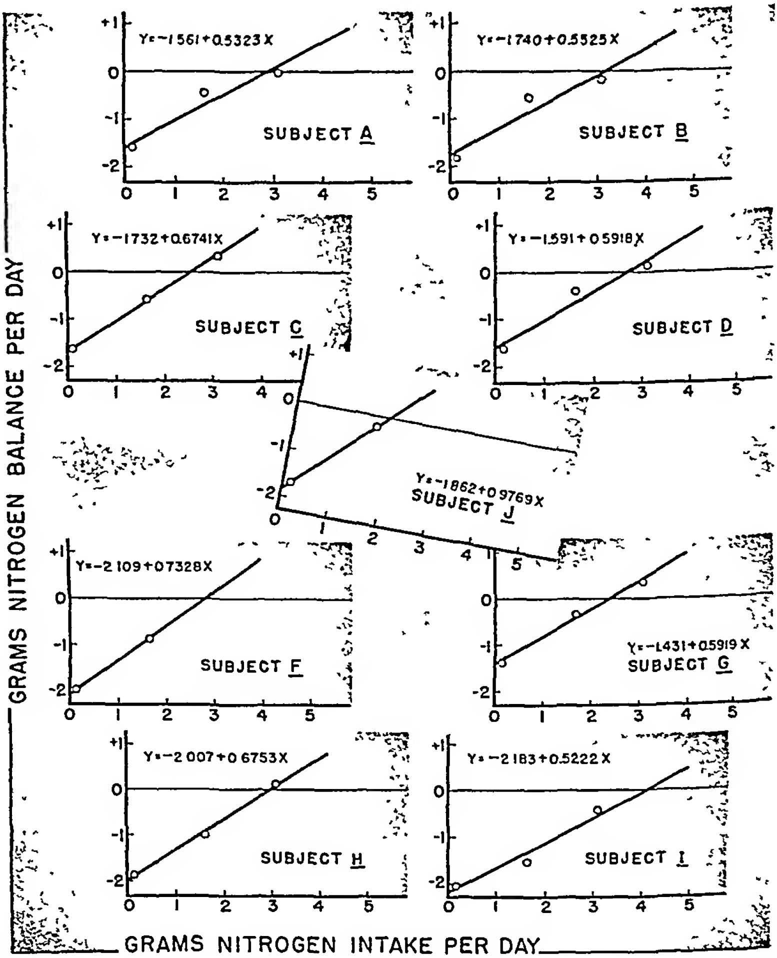

Fig. 1 Individual nitrogen balance data used in estimating the requirements of 9 college women on mixed diets are plotted on the above graphs. Regression lines fitted to the data by the method of least squares and the equations for these lines appear on each. The diet contained mixed proteins in amounts to furnish 70% of the protein from cereal.

brium with no consistently positive balance as was the case with the other subjects. This error in estimation is not surprising, since the estimate was based on two instead of three nitrogen intakes. When the intake for this subject was increased at the 8th week positive balances consistently resulted. The negative balance recorded for subject D during the 8th week can be accounted for in part by poor fecal separation as evidenced by a low value (0.436 gm nitrogen) in week 7 followed by a high value (0.944 gm nitrogen) in week 8.

TABLE 4

Estimated individual nitrogen needs on a 70% cereal protein diet

SUBJECT	PROTEIN INDEX [1]	NITROGEN FOR "ADULT GROWTH"	NITROGEN REQUIREMENT [2]	DAILY PROTEIN REQUIREMENT		
				Per person [3]	Per m^2	Per 1000 basal cal.
	%	*gm/day*	*gm/day*	*gm*	*gm*	*gm*
A	53.2	1.14	5.08	31.7	21.4	25.2
B	55.2	1.37	5.63	35.2	19.8	23.7
C	67.4	1.19	4.33	27.1	17.5	22.6
D	59.2	1.27	4.83	30.2	18.3	24.3
F	73.3	1.51	4.94	30.9	15.8	21.0
G	59.2	1.12	4.31	26.9	18.6	26.6
H	67.5	1.17	4.70	29.4	19.3	24.5
I	52.2	1.34	6.75	42.2	24.3	33.8
J	(97.7)	(1.37)	(3.31)	(20.7)	(11.6)	(16.6)
K [4]	65.0	1.37	5.13	32.0	18.0	23.8
Average (except J) [5]	61.4	1.28	5.08	31.7	19.2	25.1

[1] Nutritive value as indicated by the *b* values of the regression equations (fig. 1). This is equivalent to the coefficient of true digestibility $\times$ biological value.

[2] Calculated by solving for Y in the $Y = a + bX$ equation when $X =$ gm nitrogen needed for "adult growth."

[3] Nitrogen requirement $\times$ 6.25.

[4] Subject K did not participate during periods 1 and 2. The average nutritive index of the other 9 subjects was used as the *b* value and her total nitrogen excretion on the low nitrogen diet as the *a* value, thus giving an approximated equation of $Y = -1.961 + 0.6500\,X$, from which the daily nitrogen requirement of 5.126 gm was estimated.

[5] Estimated nitrogen requirements of J appear too low on the basis of continued negative nitrogen balances when the estimated quantities were fed.

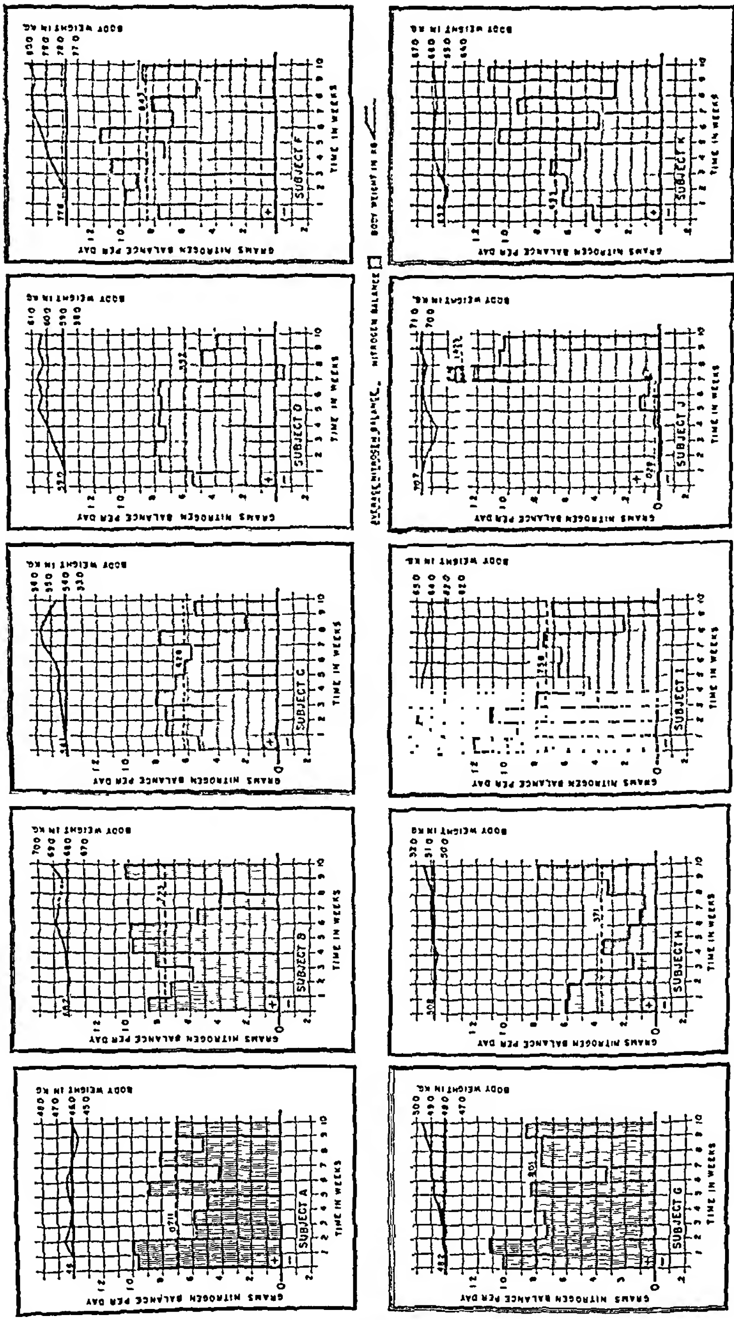

Fig. 2 Average body weights and average daily nitrogen balance (7-day balance periods) for 10 subjects fed estimated protein requirements for 10 weeks (note data are missing during weeks 3 and 8 for subjects A and B respectively).

Weekly hemoglobin values and erythrocyte counts for period 4, presented in table 5, showed some intra-subject variation but no upward or downward trend throughout the whole period. The average 10-week values for the 10 subjects ranged from 12.3 to 14.0 gm per 100 ml for hemoglobin and 4.39 and 4.99 million per mm³ for erythrocyte counts. These hemoglobin and erythrocyte values are within the normal range cited by Ohlson et al. ('44). These authors reported

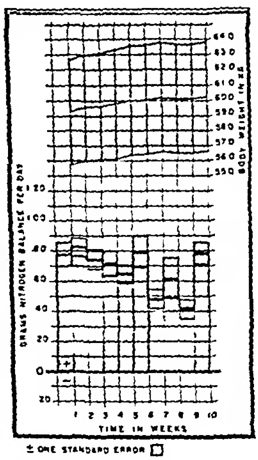

Fig. 3 Average of data shown in figure 2 for all subjects except J, together with the standard error of each mean.

hemoglobin values between 12.2 and 14.6 for 72% and erythrocyte counts of 4.18 to 4.95 for 71% of their cases. In the present study the white cell counts and packed cell volumes remained constant and within normal levels through the 10 weeks.

The performance tests at the beginning showed no consistent difference between the protein and control subjects, nor was there any indication of change in function according to these criteria after 10 weeks on the 70% cereal diet.

DISCUSSION OF RESULTS

The constancy of the apparent nitrogen stores as evidenced by the continued positive balances over the 10-week period

suggests that the estimates made by the short time experimental periods supplied ample protein, except for one subject (J). Further study of the data reveals that the average apparent nitrogen retention (average positive balance) is 0.37

TABLE 5

Weekly hemoglobin values and erythrocyte counts of 10 subjects for period 4

SUBJECT	WEEKS										Average
	1	2	3	4	5	6	7	8	9	10	
	Hemoglobin (gm/100 ml)										
A	13.1	13.0	14.1	14.0	13.4	13.6	14.0	14.8	14.2	14.0	13.8
B	13.6	14.1	13.7	13.4	13.7	14.0	14.1	...	12.2	13.9	13.6
C	14.0	12.9	12.6	12.8	13.2	12.7	12.5	12.2	13.7	13.4	13.0
D	13.0	14.0	12.8	12.8	12.8	12.9	13.3	14.0	13.5	13.7	13.3
F	13.6	13.9	13.3	14.1	14.2	14.0	14.3	14.0	13.6	14.0	13.9
G	11.8	13.1	12.0	12.1	12.3	12.1	12.2	12.4	12.7	12.0	12.3
H	13.7	14.1	13.2	13.7	13.9	13.9	13.9	15.1	14.6	14.3	14.0
I	13.6	13.2	13.9	13.6	13.9	13.2	13.8	14.4	13.7	13.6	13.7
J	12.1	12.5	13.4	13.2	13.3	12.8	13.2	13.7	13.1	14.1	13.1
K	13.7	14.1	13.7	13.6	13.2	13.0	13.5	13.4	13.0	13.9	13.5
Average	13.2	13.5	13.3	13.3	13.4	13.2	13.5	13.8	13.4	13.7	
	Erythrocyte count (millions per mm³)										
A	4.86	4.79	4.92	4.90	4.49	4.71	4.72	5.02	5.00	4.97	4.83
B	4.73	4.85	4.93	5.12	4.71	4.87	4.84	...	4.71	4.98	4.86
C	4.66	4.19	4.36	4.49	4.86	4.55	3.80	4.00	4.44	4.56	4.39
D	4.62	4.76	4.77	4.83	4.77	4.74	4.84	4.98	4.83	4.80	4.79
F	4.68	4.71	4.90	4.84	4.83	4.85	4.85	4.75	4.86	4.89	4.82
G	4.63	4.69	4.65	4.47	4.69	4.77	4.69	4.76	4.42	4.50	4.63
H	4.94	4.73	4.89	5.23	4.92	5.10	4.86	5.25	4.89	5.13	4.99
I	4.66	4.68	4.61	4.80	4.81	4.81	4.79	4.89	4.68	4.72	4.74
J	4.82	4.84	4.79	4.98	4.96	4.86	4.71	5.10	4.65	4.73	4.84
K	4.93	4.86	4.57	4.72	4.65	4.75	4.56	4.51	4.32	4.53	4.64
Average	4.75	4.71	4.74	4.84	4.77	4.80	4.67	4.81	4.68	4.78	

gm per square meter of surface area, whereas the figure used in constructing the individual protein need was 0.77 gm. This latter figure was derived from studies on men fed about 90 gm of protein daily continuously for 220 days (Grindley, '12). The indicated lower adult growth need of the girls may

have been apparent but not real, due either to an accumulated deficit of nitrogen during preceding periods or to the fact that the estimated protein need was too low to allow maximum nitrogen retentions. It is also possible that a combination of these factors may have operated. The continued positive deviation from equilibrium indicates to the authors that there is an "adult growth" need for protein nitrogen the exact amount of which has not been conclusively demonstrated in this experiment.

It is interesting to speculate as to the extent to which accumulated nitrogen deficits may have accounted for the continued positive balance. The deficits were approximated by calculating the algebraic sum of the observed and estimated[8] nitrogen balances which accumulated from the end of the Christmas holiday to the beginning of the 10-week period. The accumulated positive balances used in making this estimation were obtained by totalling the balances during the 10-week period. Accumulated positive nitrogen balances during the 10-week period were considerably greater than the nitrogen deficits in 7 of the 8 subjects for whom data were available, the average positive balance for these 7 being 51 and the negative deficit 30 gm. Subject H, the exception, had an estimated deficit of 33 and a positive storage of only 26 gm.

These data are not sufficiently conclusive to postulate a sex difference in adult growth needs, since the possibility that a higher intake of the same protein might have induced greater positive balances has not been tested. One should not ignore, on the other hand, the possibility that some of the subjects might have shown the same degree of positive balance on lower protein intakes.

The average estimated daily protein need of 31.7 gm (all subjects but J) amounts to 3.1 gm nitrogen per square meter. This is slightly above the recommendation of Hegsted et al.

[8] Estimations were essential for the first days of the period, since fecal analyses were not made. Average fecal values assayed for the last portion of the period were applied to the beginning. For the two days in which the subjects received 50 gm of protein an average storage of 4.0 gm was allowed.

('46), who estimated a nitrogen need of 2.9 gm per square meter when the nitrogen was supplied by an all-vegetable diet in which 62% of the protein was of cereal origin. A somewhat lower value, 2.4 gm, was suggested when the same diet was used and one-third the protein of each food was replaced by meat. Sherman ('20) suggests a minimum requirement of 0.5 gm protein per kilogram of body weight. When the data herein reported are expressed on a body weight basis a similar value, i.e., 0.55 gm protein per kilogram, is obtained. Neither Hegsted et al. nor Sherman considered the needs for adult growth in their estimates. If the present study had omitted such needs, the average daily protein requirement would have been 8 gm less (6.25 $\times$ 1.28, average daily nitrogen requirement for adult growth). Using regression lines of nitrogen balances on nitrogen intakes for subjects on typical self-chosen dietaries, Leverton and Marsh ('42), in a study of 70 college women, proposed a minimum daily protein requirement of 50 gm per person, and Leitch and Duckworth ('37), from a compilation of published data, suggested 48.2 to 52.4 gm as the average daily requirement. Both of these suggestions are considerably above the daily requirement of 31.7 gm indicated by the present study.

In the case of subject K, a fairly satisfactory estimate was made of the nitrogen need by using average utilization and surface area data. This was evidenced by continuous retention of nitrogen in amounts similar to those retained by other subjects.

The protein index of the protein mixture (biological value times coefficient of true digestibility) averages 61.4 ± 2.4 [9] for all subjects except J. This compares well with the value of 58.6 ± 5.9 for 5 subjects on a mixed diet reported by Bricker et al. ('45).

The constancy of the hemoglobin and erythrocyte counts throughout the 10-week period shows that the diet was adequate for maintenance of cell color and number. Leverton

[9] In this instance, and in all other cases, the value defining the significance of the mean is the standard error.

et al. ('44) have suggested that for hemoglobin regeneration intakes of at least 75 gm of protein daily are desirable, with the additional recommendation that generous amounts of animal protein be included. Obviously the process of hemoglogin maintenance should not require as generous amounts of protein as does hemoglobin regeneration.

Statistical evaluation of the data on performance tests showed no deterioration of function during the 10-week dietary period for either the protein or the control groups. In those tests where consistent improvement was noted, i.e., in memory, reading, proofreading, and clerical tests, the degree of change was not significantly different [10] between the protein and control subjects.

The initial performances of the two groups of subjects on the ergometer test were not significantly different when compared by the method of Fisher for unpaired data (Fisher, '46). Average initial scores with their standard errors were 124.8 ± 19.0 and 176.8 ± 16.4 seconds for protein and control groups, respectively. Similar comparisons for the final scores of 140.0 ± 17.2 and 159.9 ± 16.0 seconds also revealed no real difference. Application of the method of Student ('25) for the analysis of paired data to initial and final endurance scores of the protein and the control groups revealed no significant trends in muscular endurance during the 10-week period of observation.

The data for 4 of the tests — critical fusion frequency of flicker, speed of tapping, coordination time, and manual steadiness in holding a static position — were evaluated by expressing them as coefficients of comparison according to the method of Keeton et al. ('46). These coefficients are the differences between initial and final scores expressed as a multiple (or fraction) of the standard deviation for all subjects on the initial test. In none of the 4 tests mentioned above was the coefficient of comparison significantly different for the two groups of subjects and consequently the coefficients of scoring,

[10] Probability of a chance outcome was greater than 0.05.

which represented the average coefficient of comparison for each group, showed an insignificant difference between the protein and control subjects.

For the remaining performance tests (proofreading, memory, code learning, reading, and Rohrschach), the change from the initial to the final score was expressed as a percentage of the initial score. These per cent scores were then combined and compared by the method of Fisher ('46) for unpaired groups of data. The results of this comparison showed insignificant differences in all instances. Thus, though both groups had uniformly improved in all of the tests except the Rohrschach, the rate of improvement appeared to be unaffected by the dietary treatments imposed.

SUMMARY AND CONCLUSIONS

Ten college girls, 19 to 30 years of age, cooperated in a study of the protein needs of adults. During an experimental period of 105 days, divided into 4 periods, the following were determined: (1) nitrogen balances on a low protein diet; (2) individual protein needs for nitrogen equilibrium and estimated "adult growth" needs when subjects received a diet in which 70% of the protein was of cereal origin; and (3) adequacy over a 10-week period of these estimated protein needs for maintenance of nitrogen equilibrium and physical well-being (as indicated by three psychomotor tests, 5 tests of preceptual and intellective function, and one test of physical endurance).

The results were as follows:

1. The average protein index (biological value $\times$ true digestibility) of the protein mixture composed of beef, bread, 20% cream, oatmeal and potatoes was 61.4 ± 2.4 for 9 subjects for whom satisfactory data were available.

2. The quantities of protein from the above protein mixture needed for nitrogen equilibrium plus the amounts calculated to be needed for "adult growth" were found to be 31.7, 35.2, 27.1, 30.2, 30.9, 26.9, 29.4, 42.2, and 20.7 gm per day for each of 9 subjects, with an average of 30.5. A re-

quirement of 32.0 gm was estimated for a 10th subject on the basis of average utilization figures.

3. Nine of the 10 subjects were in positive nitrogen balance throughout the 10-week period during which the estimated amounts of protein were fed. The other subject, for whom the smallest indicated requirement was secured in the previous periods, showed some negative balances until the intake was increased.

4. Hematologic data and results of performance tests failed to reveal any deterioration of physiological functioning during the 10 weeks on estimated protein requirement levels.

It may be concluded from these studies: (1) that for diets of the type used, containing predominant proportions of cereal proteins, the average daily requirement for adult maintenance and growth is 31.7 ± 1.6 gm per person or 25 gm per 1000 cal. of basal heat; and (2) that the protein requirements computed from experimental periods of such length that the urinary nitrogen attains a relatively constant level are valid for periods of at least 70 days.

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THE NEW VITAMIN A REFERENCE STANDARD AND ITS USE IN EVALUATING THE VITAMIN A POTENCY OF FISH OILS[1,2]

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ONE FIGURE

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Ever since vitamin A became recognized as an essential in the normal nutrition of man and of domesticated animals, attempts have been made to develop methods which may be used to measure this nutrient quantitatively. The methods of vitamin A analysis developed thus far have been of three general types; namely, biological, physical, and chemico-physical. Since these methods are either expensive, time-consuming, or lacking in specificity, there has been a continuous attempt to improve them and to develop new methods of assay. That the problem has not been satisfactorily solved is illustrated by recent reviews of existing methods of vitamin A analysis (Association of Vitamin Chemists, Inc., '47; Dann, '47; and Nelson and DeWitt, '47). In all probability, some of the difficulty encountered in the past has been due to the vitamin A standards then in use.

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[2] Some of the data were taken from the thesis of the senior author submitted to The Graduate School of The Pennsylvania State College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Gridgeman ('44) has presented many objections to the use of the U.S.P. Reference Cod Liver Oils (no. 1 and no. 2) and of the International Standard β-carotene as vitamin A standards. More recently evidence has appeared in the literature concerning variability of the potency of different bottles of the U.S.P. Reference Cod Liver Oil no. 2 (U. S. D. A., '47). However, in 1943, U.S.P. Reference Cod Liver Oil no. 2 was replaced by U.S.P. Reference Cod Liver Oil no. 3, which in turn has recently been replaced by a new U.S.P. Vitamin A Reference Standard (crystalline vitamin A acetate dissolved in cottonseed oil, U.S.P. Vitamin Advisory Board, '47b). Furthermore, the factor used in converting physical data to biological potency has been a source of confusion. The two generally accepted conversion factors are 2000 (in the United States) and 1600 (in England).

In view of these facts, it seemed highly desirable that the relationship between the International Standard (β-carotene), U.S.P. Referencce Cod Liver Oil no. 3, the new U.S.P. Reference Standard, and pure crystalline vitamin A acetate be established through simultaneous biological assays. To clarify the situation further with respect to conversion factors, it also appeared desirable that vitamin A assays be conducted simultaneously on a number of representative commercial fish oils. It was with these objectives in mind that the experiments herewith reported were initiated.

EXPERIMENTAL PROCEDURES

In order to correlate spectrophotometric with biological data, the extinction coefficients were determined for each of the vitamin A standards and for each of the fish oils by means of the Beckman quartz spectrophotometer, using isopropanol as the solvent.

The vegetable oil used to dilute the various vitamin A supplements preliminary to feeding was a refined cottonseed oil having a peroxide value of 2.4 (milli-equivalents of peroxide oxygen per 1000 gm of oil) as determined by the method of Greenbank and Holm ('34). The vitamin A supplements were

made up in 100 ml portions and stored in several small, glass-stoppered, amber-colored bottles, each of which was flushed with CO_2 after the addition of the solution. In this connection it should be stated that the β-carotene solutions were prepared by first dissolving the weighed amount of International Standard β-carotene in 2 ml of C. P. benzene and then adding the oil. Since the benzene was not evaporated from the oil, its final concentration in the assay solutions was 0.38%, 0.58% and 0.77% in the solutions containing 10, 15, and 20 I.U. per milliliter, respectively. The dilute solutions of vitamin A and β-carotene used in the biological assays were stored at 5°C. during the feeding period. These solutions were brought to room temperature at the time of feeding and then immediately returned to the refrigerator. It was found that vitamin A and β-carotene were stable over a period of at least two weeks when the oil solutions were handled in the above manner. The stability of the vitamin A in the assay solutions was checked at frequent intervals by adding 9 ml of the Carr-Price ('26) reagent to 1 ml of the oil solution and determining the resulting $L^{1\%}_{1\,cm}$ —620 mμ value. The stability of carotene in similar solutions was checked by adding 10 ml of petroleum ether to 1 ml of the oil solution and determining the $L^{1\%}_{1\,cm}$ —440 mμ value. These determinations were made by means of the Evelyn photoelectric colorimeter.

The biological assay procedure used was essentially that set forth by the U. S. Pharmacopoeia Vitamin Advisory Board ('47a), except that the various standards were fed at multiple levels. Rats from our own stock colony were from 21 to 28 days of age and weighed from 40 to 50 gm when placed on the vitamin A-deficient diet. The rats purchased from a biological supply house were of unknown age but ranged from 40 to 50 gm in weight when placed on the vitamin-deficient diet. All test animals were maintained in individual, all-metal cages during both the depletion and curative periods.

The basal vitamin A-free diet consisted of 18% casein (hot alcohol extracted), 65% corn starch (dextrinized), 8% yeast,[4]

[4] Anheuser-Busch Strain G, irradiated.

5% hydrogenated vegetable oil,[5] and 4% salt mixture.[6] When the test animals had been depleted of their body reserves of vitamin A, they were allocated to the various assay groups in such a manner as to secure the best possible distribution by litter, sex, body weight, and state of depletion. The feeding of the vitamin A supplements was started on the day the animals were assigned to the respective groups and was continued for a 4-week period. The oily solution of the supplements was fed in small porcelain fraction cups at the rate of 0.1 ml per day. In order to insure immediate consumption of the vitamin A fractions, one or two drops of a sterilized solution of rice polish extract (20% solution of desiccated rice polish concentrate) was added to the oil supplements in each fraction cup. The biological assays were carried out in two comparable series, i.e., about half of the total number of animals were used during the first period and after the assays on these had been completed other comparable groups of animals were used. The condensed results of these studies are presented in the tables and figures which appear in this paper.

RESULTS AND DISCUSSION

Extinction coefficients of the vitamin A standards

The extinction coefficients of each of the standards used in the biological assays are presented in table 1. A comparison of these values with those reported in the literature is of interest. Oser et al. ('43) reported $E^{1\%}_{1\,cm}$ —325 mμ values for the whole and unsaponifiable fractions of U.S.P. Reference Cod Liver Oil no. 3 in isopropanol to be 1.14 and 0.98, respectively. Since our values were considerably lower than these and since similar instruments and solvents were used in the two investigations, it appears that the oil lost some of its vitamin A potency during the interim period. When one takes into consideration the matter of solvent differences the values obtained in these studies for the U.S.P. vitamin A

[5] Crisco.

[6] McCollum's no. 185.

acetate in oil (5.24 and 5.25) compare favorably with the mean values (5.37 and 5.28) for the whole and unsaponifiable fractions, respectively, obtained in connection with the recent U.S.P. collaborative study (U.S.P. Vitamin Advisory Board, '47a). The value of 1520 obtained for the crystalline vitamin A acetate prepared in this laboratory is somewhat higher than the value (1510) reported by Baxter and Robeson ('42) for this ester of the vitamin when dissolved in ethyl alcohol, but is the same as that reported by Guerrant et al. ('48).

TABLE 1

Extinction coefficients of the vitamin A standards used in the biological assays

STANDARD	SOLVENT	WAVE LENGTH	$E_{1\,cm}^{1\%}$
U.S.P. Reference Cod Liver Oil no. 3	Isopropanol	325 mμ	0.914
	Isopropanol	325 mμ	0.817 [1]
New U.S.P. Vitamin A Reference Standard	Isopropanol	325 mμ	5.24
	Isopropanol	325 mμ	5.25 [1]
Crystalline vitamin A acetate prepared in this laboratory	Isopropanol	325 mμ	1520
International Standard (β-carotene)	Petroleum ether	450 mμ	2300

[1] Nonsaponifiable fraction.

Extinction coefficients reported in the literature for pure β-carotene (Gridgeman, '44; Devine et al., '45; Guerrant et al., '48) are generally somewhat higher than the coefficient found for the International Standard used in this investigation and this suggests that the latter is no longer the purest form of β-carotene obtainable (Van Genderen and Van Eekelen, '44; Wilkinson, '41).

Results of the biological assays

Some of the pertinent biological data obtained during the course of assaying the vitamin A standards and the 27 representative fish oils used are presented in tables 2, 3, and 4.

TABLE 2

Comparative biological responses of the various groups of rats used in assaying the vitamin A standards and the 27 representative fish oils

ASSAY GROUP	SOURCE OF VITAMIN A	ESTIMATED DOSAGE [1]	MALES	FEMALES	AVERAGE INITIAL WEIGHT	DEPLETION PERIOD		AVERAGE GAIN IN WEIGHT DURING ASSAY				
						Average duration	Average weight at end	Males	Females	Males and females: Unweighted [2]	Males and females: Weighted [3]	Standard deviation [3]
		units/day	*no.*	*no.*	*gm*	*days*	*gm*	*gm*	*gm*	*gm*	*gm*	
1	New U.S.P. Vitamin A Reference Standard	0.75	7	8	45	27.9	103	16.6	22.8	19.9	19.7	11.2
2	New U.S.P. Vitamin A Reference Standard	1.0	7	6	46	27.8	106	19.6	23.2	21.2	21.4	8.5
3	New U.S.P. Vitamin A Reference Standard	1.5	7	8	46	27.6	101	31.6	30.6	31.1	31.1	10.3
4	New U.S.P. Vitamin A Reference Standard	2.0	8	8	46	27.7	104	36.0	35.8	35.9	35.9	7.3
5	U.S.P. Reference Cod liver oil no. 3	1.0	5	8	44	27.4	100	10.0	26.3	20.0	18.1	13.6
6	U.S.P. Reference Cod liver oil no. 3	1.5	7	7	46	27.8	102	23.3	30.6	26.9	26.9	9.3
7	U.S.P. Reference Cod liver oil no. 3	2.0	8	8	46	29.6	104	29.3	27.5	28.4	28.4	9.6
8	International Standard	1.0	3	7	44	26.7	94	25.3	23.3	23.9	24.3	7.9
9	International Standard	1.5	8	8	45	27.2	104	36.5	24.5	30.5	30.5	11.0
10	International Standard	2.0	8	7	46	27.7	108	41.6	32.6	37.4	37.1	9.0
11	Vitamin A acetate prepared in this laboratory	1.0	7	6	46	28.4	106	21.1	25.5	23.2	23.3	7.7
12	(Vitamin A acetate prepared in this laboratory)	1.5	7	8	44	27.9	101	32.1	30.9	31.5	31.5	6.6
13	Negative control		6	6	45	27.6	102		...			
14	Swordfish liver oil, domestic [4]	1.5	4	7	44	29.4	108	32.5	22.5	28.5	27.5	7.2
15	Swordfish liver oil, imported [4]	1.5	5	7	46	29.1	104	37.2	28.1	31.9	32.7	12.5
16	Skip Jack liver oil [4]	1.5	4	7	46	29.0	104	31.3	21.9	25.3	26.6	10.7
17	Skip Jack liver oil, imported [4]	1.5	4	7	46	28.5	108	25.8	29.6	28.2	27.7	7.9
18	Bluefin tuna liver oil [4]	1.5	3	6	45	29.4	106	27.3	21.8	23.7	24.6	6.7
19	Bluefin tuna liver oil, imported [4]	2.0	3	7	45	28.5	110	31.0	26.6	27.9	28.8	5.6
20	Totuava liver oil [4]	1.5	5	6	45	29.6	106	19.4	24.2	22.0	21.8	10.3
21	Totuava liver oil, imported [4]	1.5	4	7	44	29.8	102	32.3	18.1	23.3	25.2	10.4
22	Albacore liver oil, no. 1 [4]	1.5	5	7	48	29.6	112	41.2	25.3	31.9	33.2	13.4
23	Albacore liver oil, no. 2 [4]	1.5	5	6	45	30.3	108	25.8	28.5	27.3	27.2	13.7
24	Barracuda liver oil [4]	1.5	4	6	48	29.7	109	11.8	26.2	20.4	19.0	10.9
25	Black cod liver oil [4]	1.5	4	7	46	29.4	105	31.8	27.7	29.2	29.7	9.5
26	Bonita liver oil [4]	1.5	4	7	45	28.5	107	31.0	28.6	29.5	29.8	7.8
27	Halibut liver oil [4]	2.0	4	5	47	29.5	112	18.5	30.4	25.1	24.5	14.7
28	Jewfish liver oil [4]	2.0	5	6	46	29.4	111	36.6	32.8	34.5	34.7	9.2
29	Ling cod liver oil [4]	1.5	4	7	45	29.5	105	16.0	23.0	20.5	19.5	9.0
30	Mackerel liver oil [4]	1.5	4	7	45	28.0	106	34.8	24.0	27.9	29.4	10.2
31	Menuko liver oil, imported [4]	1.5	5	7	45	28.2	106	28.6	24.3	26.1	26.4	8.2
32	Sebastus Marinus liver oil [4]	1.5	5	6	46	28.0	105	26.0	40.3	33.8	33.2	12.4
33	Spearfish liver oil, imported [4]	1.5	4	6	45	28.5	107	21.0	30.7	26.9	25.9	12.2
34	Yellow Tail liver oil [4]	1.5	4	7	45	28.1	105	29.5	25.1	26.7	27.3	8.8
35	Argentine shark oil [5]	2.0	4	6	44	35.7	127	30.7	29.3	29.9	30.0	10.2
36	Dogfish liver oil [5]	2.0	4	6	45	34.9	123	45.0	30.7	36.4	37.8	10.1
37	Blue shark oil [5]	2.0	5	5	45	35.0	127	19.2	20.8	20.0	20.0	11.0
38	Fortified cod liver oil [5]	2.0	4	6	44	36.4	127	29.2	24.0	26.1	26.6	6.4
39	Soupfin shark oil [5]	2.0	4	7	45	35.8	128	25.5	30.4	28.6	28.0	5.6
40	Mexican shark oil [5]	2.0	5	6	45	34.9	125	28.8	30.2	29.5	29.5	10.9

[1] Based on spectrophotometric data.
[2] For sex.
[3] Based upon the weighted average gain in weight during assay.
[4] Furnished through the courtesy of Mead Johnson and Company, Evansville, Indiana.
[5] Furnished through the courtesy of Distillation Products, Inc., Rochester, New York.

It is to be noted that some of the oils were fed at the 1.5 unit level while others were fed at the 2.0 unit level (see table 2). This was because preliminary assays had shown the estimated potency of certain oils, based on spectrophotometric data, to be too high; hence an increased amount of some of the other oils was fed during the biological assay.

There was no evidence by the colorimetric method of assay of a loss of either vitamin A or carotene from the diluted oil solutions during a 120-day storage period under the conditions

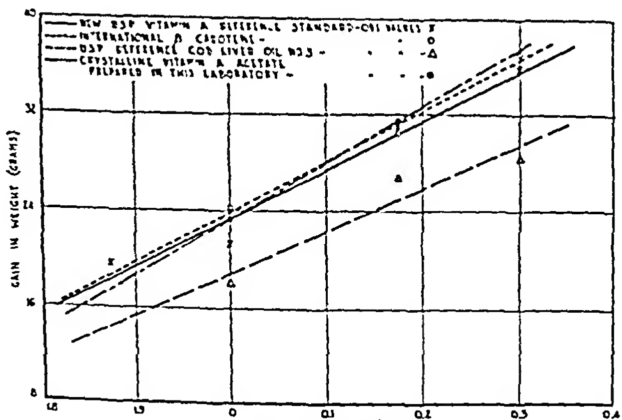

Fig. 1 Response graphs for the different vitamin A standards. The points represent the observed responses, while the lines represent the calculated responses. Here it is to be noted that U.S.P. Reference Cod Liver Oil no. 3 was definitely less effective in promoting growth, per unit of vitamin, than was the vitamin A acetate or the International Standard (β-carotene).

of these experiments. It appears, therefore, that the usual practice of preparing fresh solutions of the vitamin A standards and the vitamin A carriers at weekly intervals is not always necessary, provided a satisfactory solvent oil is used and provided the vitamin solutions are properly handled and stored.

On the basis of data presented in table 2 (groups 1 to 12, inclusive) it is possible to compare the biological responses resulting from feeding various definite amounts of the different vitamin A standards. In setting up the biological tests it

was originally planned that all test groups would be composed of equal numbers of comparable animals, but owing to the fact that some animals died before the assays were completed, the data presented in table 2 do not show this experimental design. However, in order to compensate partially for unequal sex distribution the average growth response of each assay group was weighted for sex in making the final computations. The dose-response equations were derived according to the method of Coward ('38) and by means of these equations growth responses were calculated. The calculated responses as well as the observed responses are illustrated in figure 1. The equations for the lines given are: New U.S.P. Vitamin A Reference Standard: $y = 40.5x + 23.4$; International Standard: $y = 42.1x + 23.9$; U.S.P. Reference Cod Liver Oil no. 3: $y = 46.9x + 23.3$. In each formula y equals the growth response in grams during the 28-day assay period and x equals the log (to the base 10) of the daily dose of the vitamin.

The potency of a given vitamin A standard in terms of the other standards was evaluated by substituting the calculated growth response (the growth response represented by the appropriate equation, using the 1.0, 1.5, and 2.0 unit levels, respectively) and then determining the arithmetic mean of the three values thus obtained. Thus, in terms of the International Standard (β-carotene), the new U.S.P. Vitamin A Reference Standard was found to contain 9610 I.U. per gram, the U.S.P. Reference Cod Liver Oil no. 3 to contain 1220 I.U. per gram, and the crystalline vitamin A acetate to contain 2.93×10^6 I.U. per gram. In terms of the new U.S.P. Vitamin A Reference Standard, the crystalline vitamin A acetate was found to contain 3.045×10^6 U.S.P. units per gram, while the U.S.P. Reference Cod Liver Oil no. 3 contained 1250 U.S.P. units per gram. In other words, the U.S.P. unit in the new U.S.P. Vitamin A Reference Standard was found to be 1.36 times as active biologically as was the U.S.P. unit in the former standard (U.S.P. Reference Cod Liver Oil no. 3) when this standard was assumed to contain 1700 U.S.P. units per gram. The potency observed for the U.S.P. Reference Cod Liver Oil

no. 3 (1220 I.U. per gram) is less than that reported as being the average potency (1365 I.U. per gram) obtained in the U.S.P. Vitamin A Collaborative Study of 1946–1947 (U.S.P. Vitamin Advisory Board, '47a). However, both values definitely indicate that the U.S.P. unit as found in the U.S.P. Reference Cod Liver Oil no. 3 and the International unit do not represent the same amount of biological activity. With the introduction of the new U.S.P. Vitamin A Standard, the International unit and the U.S.P. unit may be considered to be equal in biological potency. The fact that U.S.P. Reference Cod Liver Oils no. 2 and no. 3 deteriorated in biological potency during the course of their usage accounts for many of the discrepancies in the literature concerning the potency of certain vitamin A carriers, and perhaps for some of the variation in reported conversion factors.

The potency of the crystalline vitamin A acetate prepared in this laboratory compares favorably with that in the new U.S.P. Vitamin A Reference Standard. Calculated as the alcohol, the potency found for this crystalline vitamin A acetate was 3.492×10^6 U.S.P. units per gram. This value is in relatively good agreement with those of 3.181×10^6 and 3.424×10^6 I.U. per gram reported by Mead, Underhill and Coward ('39) for vitamin A alcohol when examined in the form of the carboxylate and naphthoate esters, respectively. If one multiplies 3.492×10^6 U.S.P. units per gram by the factor 1.36 indicated above, one obtains a potency for vitamin A alcohol of 4.749×10^6 U.S.P. units per gram. On the basis of these calculations the value of 4.30×10^6 U.S.P. units per gram of vitamin A alcohol as originally reported by Baxter and Robeson ('42) is perhaps explained. If the potency of 3.50×10^6 U.S. P. units per gram of vitamin A alcohol which has been reported more recently (Embree, '47; Hanze et al., '48) is based upon the present U.S.P. unit, it is in close agreement with the potency observed in this investigation. It is evident from the above studies on the relationships among the different vitamin A standards, and from stability and spectral absorption studies reported elsewhere of solutions

TABLE 3

Comparative potencies of the various vitamin A carriers, as measured by the spectrophotometric method and by the biological method of assay, and calculated conversion factors

ASSAY GROUP	SOURCE OF VITAMIN A	$E^{1\%}_{1\,cm}$ — 325 mμ[1]	CALCULATED POTENCY $E^{1\%}_{1\,cm}$ — 325 mμ × 2000	BIOLOGICAL POTENCY FOUND IN TERMS OF[2] U.S.P. Reference Cod Liver Oil no. 3	New U.S.P. Vitamin A Reference Standard	CONVERSION FACTOR BASED UPON U.S.P. Reference Cod Liver Oil no. 3	New U.S.P. Vitamin A Reference Standard
				units/gm			
1	New U.S.P. Vitamin A Reference Standard	5.25	10,500	13,600		2590	
2	U.S.P. Reference Cod Liver Oil no. 3	.817	1,634		1,250		1530
3	Crystalline vitamin A acetate	1520.0[3]	3,040,000	4,141,000	3,045,000	2720	2000
4	Swordfish liver oil, domestic	80.8	161,600	177,500	130,500	2200	1620
5	Swordfish liver oil, imported	62.7	125,400	184,600	135,700	2940	2160
6	Skip Jack liver oil	20.8	41,600	43,500	31,980	2090	1540
7	Skip Jack liver oil, imported	25.1	50,200	56,000	41,170	2230	1640
8	Bluefin tuna liver oil	29.0	58,200	54,100	39,760	1870	1370
9	Bluefin tuna liver oil, imported	8.05	16,100	14,300	10,520	1780	1310
10	Totuava liver oil	28.6	57,200	45,450	33,420	1590	1170
11	Totuava liver oil, imported	74.7	149,400	111,600	106,300	1490	1420
12	Albacore liver oil, no. 1	35.5	71,000	108,300	79,600	3050	2240
13	Albacore liver oil, no. 2	7.69	15,380	16,620	12,220	2160	1590
14	Barracuda liver oil	42.5	85,000	57,700	42,420	1360	1000
15	Black cod liver oil	40.3	80,600	100,150	73,850	2490	1830
16	Bonita liver oil	31.4	62,800	78,780	57,930	2510	1850
17	Halibut liver oil	9.53	19,060	13,210	9,710	1390	1020
18	Jewfish liver oil	321.0	642,000	797,200	586,200	2480	1830
19	Ling cod liver oil	40.0	80,200	56,110	41,260	1400	1030
20	Mackerel liver oil	41.6	83,200	102,200	75,160	2460	1810
21	Menuke liver oil, imported	71.7	143,400	148,600	109,300	2070	1520
22	Sebastus Marinus liver oil	8.89	17,780	27,120	19,940	3050	2240
23	Spearfish liver oil, imported	51.0	102,000	102,200	75,130	2000	1470
24	Yellow Tail liver oil	20.1	40,200	43,780	32,190	2180	1600
25	Argentine shark oil	15.4	31,000	30,700	22,560	1990	1470
26	Dogfish liver oil	4.56	9,100	13,900	10,220	3050	2240
27	Blue shark oil	10.7	21,400	12,200	8,970	1140	840
28	Fortified cod liver oil	1.99	4,000	3,280	2,410	1650	1210
29	Soupfin shark oil	63.4	127,000	113,000	83,100	1780	1310
30	Mexican shark oil	17.4	35,000	33,800	24,840	1940	1430
	Arithmetic mean of conversion factors for the fish oils					2100	1550
	Logarithmic mean of conversion factors for the fish oils					2040	1500

[1] Nonsaponifiable fraction dissolved in isopropyl alcohol

[2] Samples 1 to 24, inclusive were assayed simultaneously against the New U.S.P. Vitamin A Reference Standard and against U.S.P. Reference Cod Liver Oil no. 3 Samples 25 to 30, inclusive, were assayed simultaneously against U.S.P. Reference Cod Liver Oil no. 3, which in turn was subsequently assayed against the New U.S.P. Vitamin A Reference Standard

[3] For the unsaponified cyrstalline vitamin A acetate

similar to the present U.S.P. Vitamin A Standard (Guerrant et al., '48; Chilcote et al., '48a, '48b) that definite progress is being made in solving some of the problems encountered in vitamin A determinations.

The potency of the different fish oils is presented in table 3. Here it is to be noted that different samples of oil from the same species of fish frequently show wide differences in $E^{1\,cm}_{1\%}$ —325 mμ value, as well as in biological potency. Whether these differences are due to the feeding habits of the respective fish or to previous handling of the oils cannot be determined from the available information.

Concerning conversion factors

Conversion factors for the vitamin A standards and for the various fish oils were calculated by dividing the potency found by bioassay by the $E^{1\%}_{1\,cm}$ — 325 mμ value obtained for the unsaponifiable fraction of the oil when dissolved in isopropanol. Furthermore, average conversion factors were calculated arithmetically and also logarithmically, as suggested by Gridgeman ('44). The relatively high conversion factors based upon U.S.P. Reference Cod Liver Oil no. 3 as the standard and the low conversion factors based on the present U.S.P. Vitamin A Reference Standard (essentially the same as the International Standard), are merely reflections of the relative biological potencies of the two standards. Inasmuch as these standards were originally supposed to contain units of equal biological value the wide discrepancy in conversion factors based on them again suggests that U.S.P. Reference Cod Liver Oil no. 3 had lost about one-fourth of its biological potency before being used.

An examination of the conversion factors presented in table 3 indicates that a single such factor is not applicable to all fish oils, a conclusion previously reached by other investigators (Coy et al., '43; Zscheile and Henry, '44). This conclusion is also suggested by a summary of the various conversion factors presented by Gridgeman ('44). A satisfactory explanation

for the frequently observed variations in conversion factors for different vitamin A carriers has not been offered up to the present time. The inherent errors of biological assay may be contributing factors, but they certainly are not sufficient to account for wide variations, which range up to 250%. The presence or absence of varying amounts of materials in vitamin carriers which interfere with spectrophotometric measurement of vitamin A seems to offer a logical explanation for part of the variation among conversion factors. However, in these and in other studies neither saponification nor chromatography was found to be uniformly effective in reducing the relative variability of the calculated conversion factors, although in general both treatments resulted in higher factors for the treated oils than were obtained for the whole oils. Another possible explanation which is receiving considerable emphasis at the present time is based on the belief that spectrographic data are reasonably reliable but that the vitamin A present in various fish oils and vitamin carriers varies in its molecular structure and also in its availability to the test animal. Such an explanation seems to be based on logical premises; however, it is of interest to note that published data are not available to show that the biological potency of fish liver oils can be increased by saponification, a treatment which tends to convert all forms of the vitamin to the alcohol form. Furthermore, no published data are available to show that conversion factors calculated from biological data obtained by feeding the nonsaponifiable residues of fish liver oils are less variable than are the corresponding factors based on the biological assay of the whole oil.

Concerning the variabilities in the conversion factors reported in the present study, there appeared to be no indication of a consistent association of high or of low conversion factors with high or low biological potencies, nor was there any definite relationship between the L-values of the assay solutions and the respective conversion factors.

Methods which may be employed to increase the accuracy of the results obtained through spectrophotometric analysis

TABLE 1

Conversion factors for vitamin A acetate and for fish oils when calculated on the basis of the $E^{1\%}_{1\,cm}$ — 325 mμ of the whole oils and of the respective nonsaponifiable fractions and of the bone meal chromatographed residues

ASSAY GROUP	SOURCE OF VITAMIN A	BIOLOGICAL POTENCY OF WHOLE OIL	CONVERSION FACTOR BASED ON: Whole oil	Nonsaponifiable fraction	Bone meal chromatographed fraction
		U S P units/gm			
1	New U S P. Vitamin A Reference Standard	10,000			
2	U S P. Reference Cod Liver Oil no 3	1,250	1390	1510	1710
3	Crystalline vitamin A acetate	3,015,000		2000 [1]	
4	Swordfish liver oil, domestic	110,500	1590	1620	1610
5	Swordfish liver oil imported	135,700	2080	2160	2190
6	Skip Jack liver oil	31,980	1110	1510	1560
7	Skip Jack liver oil, imported	11,170	1610	1610	1760
8	Bluefin tuna liver oil	19,760	1290	1370	1570
9	Bluefin tuna liver oil, imported	10,520	910	1110	1140
10	Totuava liver oil	33,120	1120	1170	1220
11	Totuava liver oil, imported	106,100	1100	1120	1600
12	Albacore liver oil no 1	79,600	2220	2310	2410
13	Albacore liver oil, no 2	12 230	1110	1590	1620
14	Barracuda liver oil	12 120	980	1000	1970
15	Black cod liver oil	71,850	1830	1830	1900
16	Bonita liver oil	57,930	1810	1850	1920
17	Halibut liver oil	9,710	940	1020	1110
18	Jewfish liver oil	586 200	1820	1830	2130
19	Ling cod liver oil	11 260	1000	1070	1050
20	Mackerel liver oil	75 160	1720	1810	1880
21	Menuko oil, imported	109,300	1160	1520	1620
22	Sebastus Marinus liver oil	19 910	2120	2210	2300
23	Spearfish liver oil, imported	75,110	1100	1170	1710
24	Yellow Tail liver oil	72,190	1560	1600	1670
25	Argentine shark oil	22 560	1170	1170	1510
26	Dogfish liver oil	10 220	2170	2210	2350
27	Blue shark oil	8 970	910	810	1010
28	Fortified cod liver oil	2,110	1210	1210	1710
29	Soupfin shark oil	83,100	1110	1110	1790
30	Mexican shark oil	21 810	1300	1110	1550
	Arithmetic mean conversion factor for the fish oils		1180	1530	1630
	Logarithmic mean conversion factor for the fish oils		1170	1500	1620

[1] Based on the $E^{1\%}_{1\,cm}$ — 325 mμ of the crystalline ester.

for vitamin A have been suggested in recent years (Morton and Stubbs, '46, '47, 48; Müller and Reinert, '46). The latter workers claim that by applying an aluminum oxide adsorption technique previous to spectrophotometric measurements, conversion factors of 1700 are obtained; whereas, if the oil is not first chromatographed by this technique, conversion factors below 1000 result. This is partially borne out by the data presented in table 4, where the mean conversion factor for the bone meal chromatographed residues (Glover, Goodwin and Morton, '47) from the various vitamin A carriers used in the present studies was found to be higher than the mean conversion factor for the whole oils or for the nonsaponifiable residues.

Morton and Stubbs ('48) have reported on a technique whereby spectrophotometric measurements are made at different wave lengths and certain corrections are made for irrelevant absorption. These authors claim that by this procedure a conversion factor of 1800 is obtained, whereas a factor of 1600 is obtained when the calculations are based on the gross absorption measurements. It appears, however, that further investigations of this type must be carried out before the biological potencies of all fish oils can be evaluated by physical means.

SUMMARY

A comparison has been made of the extinction coefficients and the relative biological potencies of U.S.P. Reference Cod Liver Oil no. 3, the new Vitamin A Reference Standard, crystalline vitamin A acetate, and the International Standard. In addition, 27 representative fish oils have been assayed biologically against these standards and conversion factors calculated. The results obtained in this investigation may be summarized as follows:

1. The extinction coefficients of the vitamin A standards employed were found to be:

(a) U.S.P. Reference Cod Liver Oil no. 3: whole oil — 0.914; unsaponifiable fraction — 0.817.

(b) New U.S.P. Vitamin A Reference Standard: whole oil — 5.24; unsaponifiable fraction — 5.25.

(c) Crystalline vitamin A acetate — 1520.

(d) International Standard (β-carotene) — 2300.

2. Stability studies conducted with vitamin A and carotene solutions prepared for feeding purposes showed these sources of vitamin A to be stable over a two-week feeding period and during 120-day storage at 5°C., provided the solutions were protected against atmospheric oxidation.

3. A comparison of the relative biological potencies of the Vitamin A Standards indicated that the present U.S.P. unit and the International unit may be considered equal. The U.S.P. unit as represented by the new U.S.P. Vitamin A Reference Standard was found to be 1.36 times as potent biologically as the U.S.P. unit represented by U.S.P. Reference Cod Liver Oil no. 3. The potency of the crystalline vitamin A acetate was found to be 3.045×10^6 U.S.P. units per gram.

4. The conversion factors calculated for 27 representative fish oils (nonsaponifiable fraction) in terms of the present U.S.P. Vitamin A Reference Standard ranged from 840 to 2240, with a logarithmic mean of 1500. Conversion factors for the same oils calculated in terms of U.S.P. Reference Cod Liver Oil no. 3 ranged from 1140 to 3050, with a logarithmic mean of 2040. These mean values (1500 and 2040) indicate the extent of the decrease in potency of the cod liver oil standard, and may suggest a partial explanation for the existing confusion regarding conversion factors. The variability of the conversion factors for the fish oils used in the present study indicates that no one single conversion factor will be readily applicable to all fish oils.

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COMPARISON OF THE ASCORBIC ACID CONTENT OF PLASMA AND OF WHOLE BLOOD AS CRITERIA OF NUTRITIONAL STATUS

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TWO FIGURES

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As is discussed in their recent papers, Roe and coworkers ('46, '47) found plasma ascorbic acid values in both man and the guinea pig higher than those of whole blood when whole blood values were 0.6 mg % or above, whereas at lower whole blood levels plasma values were lower than those of whole blood. The above authors interpreted these findings as indicating that a whole blood ascorbic acid level of 0.6 mg % is an adequate level and that an adequate intake of the vitamin is an amount which will maintain the whole blood level at or above this concentration. This interpretation would be very significant if further investigation should show that plasma ascorbic acid levels are consistently higher than those of whole blood at some specific level.

The purpose of the present investigation was to compare the ascorbic acid concentration in the whole blood with that in plasma with regard to (1) levels at which the values of plasma exceed those of whole blood and (2) the variability of ascorbic acid values under conditions of depletion and repletion.

[1] Submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in the Department of Home Economics, University of Chicago.

[2] The study of urinary excretion of ascorbic acid has been submitted by Miss Attaya in partial fulfillment of the requirements for the Master of Science degree, Department of Home Economics, Louisiana State University.

METHOD

Eight healthy young people were placed on a controlled diet and frequent determinations were made of whole blood and plasma ascorbic acid levels and of the 24-hour urinary excretion of the vitamin. The subjects were 7 students at Louisiana State University and one faculty member. All were living the usual life of science students engaged in laboratory and class activities and none was inclined to take part in strenuous athletics. Table 1 shows the sex, height, weight and age of each subject.

TABLE 1

Description of subjects and ascorbic acid dosage

CATEGORY OF INTEREST	SUBJECT							
	1	2	3	4	5	6	7	8
Sex	F	F	F	M	M	M	M	M
Height (in.)	67	67	67½	71½	69	67½	71	65½
Weight (lb.)	124	135	120	180	162	130	170	135
Age (years)	19	22	25	28	35	23	23	22
Ascorbic acid dosage								
Preliminary period (mg)	100	100	{100 200	200	200	100	100	100
Preliminary period (days)	11	11	{8 3	11	5	11	11	11
Depletion period, basal diet only (days)	25	25	25	25	25	25	25	25
Repletion period, basal diet and ascorbic acid								
100 mg morning (days)	21	21	21	21	28	28	28	28
100 mg in morning and 100 mg at supper (days)	8	8	8	8	14	14	14	14
200 mg at supper (days)	4	4	4	4				
400 mg test dose at supper (days)					1	1	1	1
Basal diet (days)	2	2	2	2	2	2	2	2

Preliminary period

During a preliminary period all subjects were given an ascorbic acid supplement in addition to their usual diet in order to start the experimental period with all individuals in a good state of nutrition with regard to this vitamin.

Depletion period

After this preliminary period the subjects were placed on a basal diet which was prepared and served in the laboratory. The diet was designed to satisfy metabolic needs in all respects except as regards ascorbic acid. The ascorbic acid content of this diet had been determined repeatedly in connection with a previous study by the senior author (Hollinger, '48) and found to average 15 mg daily with little variation.

Repletion period

Following 25 days of depletion a daily supplement of 100 mg of ascorbic acid was added to the basal diet. This supplement was taken in the morning after blood samples were obtained. Subjects 1, 2, 3 and 4 continued on this regime for three weeks, after which an additional 100 mg of ascorbic acid was provided at the evening meal for 8 days. For another 4 days the entire 200 mg supplement was taken at the evening meal. The supplement was then discontinued while determinations of whole blood and plasma ascorbic acid and the urinary excretion of the vitamin were continued two days longer.

Because the remaining 4 subjects had shown a slower rise in whole blood and plasma ascorbic acid and urinary excretion values, they continued for 28 days on the 100 mg supplement, after which an additional 100 mg of ascorbic acid were taken at the evening meal for 14 days. This regime was followed by the administration of a 400 mg test dose. Determinations of whole blood and plasma ascorbic acid were made for three successive days, and of urinary excretion for two successive days, immediately following the ingestion of the 400 mg test

dose, during which time no further supplements were taken. All changes in the intake of ascorbic acid were made on the day just preceding the taking of a blood sample.

The ascorbic acid supplements (100 and 200 mg) were selected because they were presumed to be large enough to produce rapid rises in blood values and to give ample opportunity for comparing whole blood and plasma values under conditions of rapid repletion.

During the depletion and repletion periods whole blood and plasma ascorbic acid determinations were made on Monday, Tuesday, Thursday and Friday of each week, in order to compare values for consecutive days. Blood samples were drawn immediately after breakfast, which contained no foods having appreciable amounts of ascorbic acid.

About 12 ml of blood were taken from the arm by venous puncture and transferred to a centrifuge tube containing two or three drops of 20% potassium oxalate solution to prevent coagulation. After thorough mixing and the pipetting off of an aliquot of whole blood, the remainder was centrifuged for about 45 minutes to separate cells from plasma.

Twenty-four hour urine collections were made on 5 days each week, beginning on Monday morning and ending Saturday morning. Urine was collected in brown jars containing about 2 gm of crystalline oxalic acid and 2 ml of toluene.

The method of Roe and Kuether ('43) was followed in detail for the determination of ascorbic acid in whole blood, plasma and urine.

RESULTS

Whole blood and plasma ascorbic acid values in milligrams per cent, together with the urinary excretion values in milligrams, are presented in figures 1 and 2.

When the data from the 283 comparable samples of whole blood and plasma are examined as a whole it is evident that, with the exception of 15 samples, plasma values were lower than those of whole blood. During the entire depletion period only one sample was obtained in which the plasma value was higher than that of whole blood (fig. 1 B), although in 59

samples whole blood ascorbic acid values were found to be 0.6 mg % or above.

During the repletion period plasma values were lower than those of whole blood, with 14 exceptions. In 4 samples obtained from subjects 1 and 2 early in the repletion period the ascorbic acid value of the plasma slightly exceeded that of the whole blood (fig. 1, A and B). The remaining 10 samples in which the plasma ascorbic acid value exceeded that of whole blood were obtained from subjects 1, 3, 5 and 8 near the end of the repletion period while the 200 mg supplement was being taken, and, in the cases of subjects 5 and 8, after the 400 mg test dose. These latter 10 samples were obtained at a time when the subjects were saturated, as evidenced by the excretion of one-third or more of their ascorbic acid intake. In these subjects the excess of ascorbic acid in the plasma was not consistent but occurred in one or two samples, followed by samplings in which plasma values were lower than those of whole blood. In subjects 4, 6, and 7 plasma values were lower than those of whole blood in every sample obtained during both depletion and repletion, although these subjects maintained whole blood levels of 0.6 mg % or above during the last two to three weeks of depletion. These three subjects were saturated, as evidenced by the excretion of one-third or more of their ascorbic acid intake, for two weeks before the supplement was withdrawn (fig. 1 D, fig. 2, F and G). Subject 2 excreted 100 mg or more during the entire period on the 200 mg supplement but showed no plasma ascorbic acid value higher than that of whole blood during the repletion period.

The results of the present study show no evidence of a definite ascorbic acid level at which the plasma value consistently exceeds that of whole blood. This observation is in direct contrast to the findings of Roe and coworkers ('46, 47), who reported that in most cases where whole blood ascorbic acid values were 0.6 mg % or above, the plasma concentration was higher than that of whole blood. The evidence appears rather to confirm the conclusions of Heineman ('41)

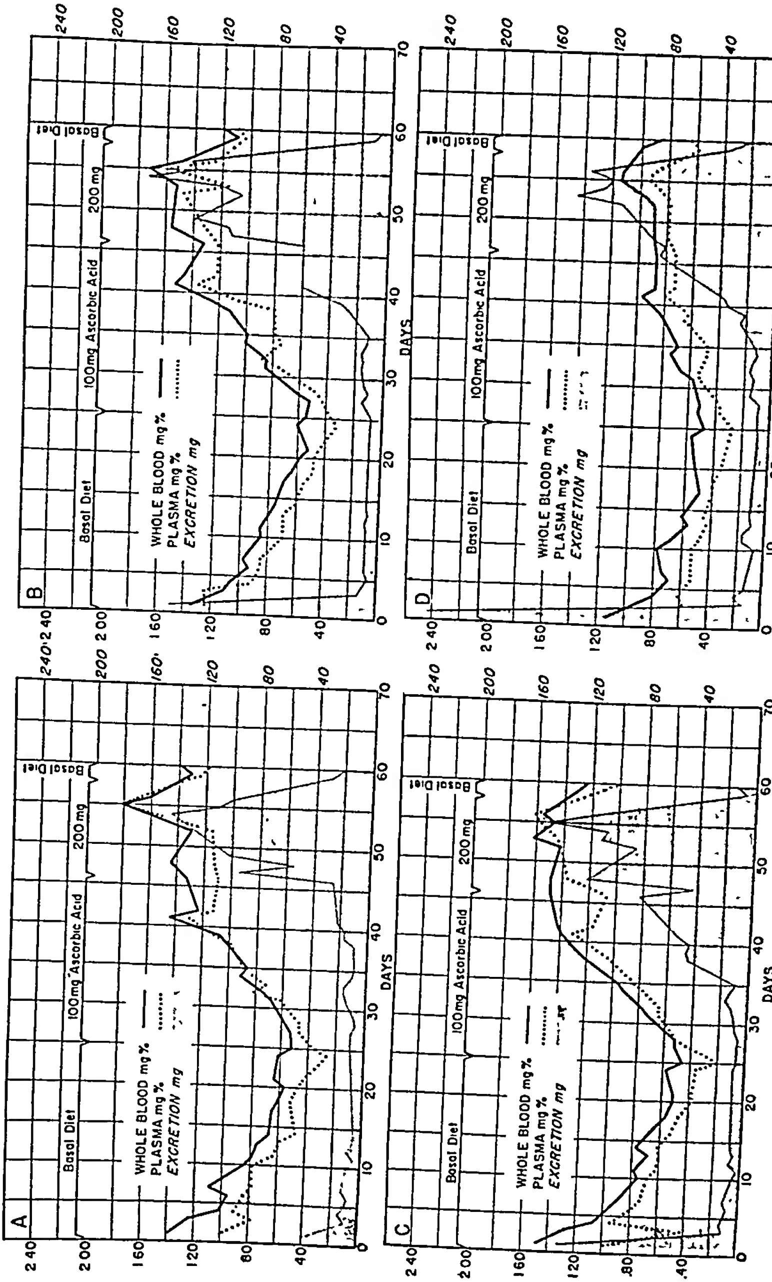

Fig. 1 A, B, C and D refer to subjects 1, 2, 3 and 4, respectively. Left ordinate, scale for plasma and whole blood. Right ordinate, scale for 24-hour urinary excretion.

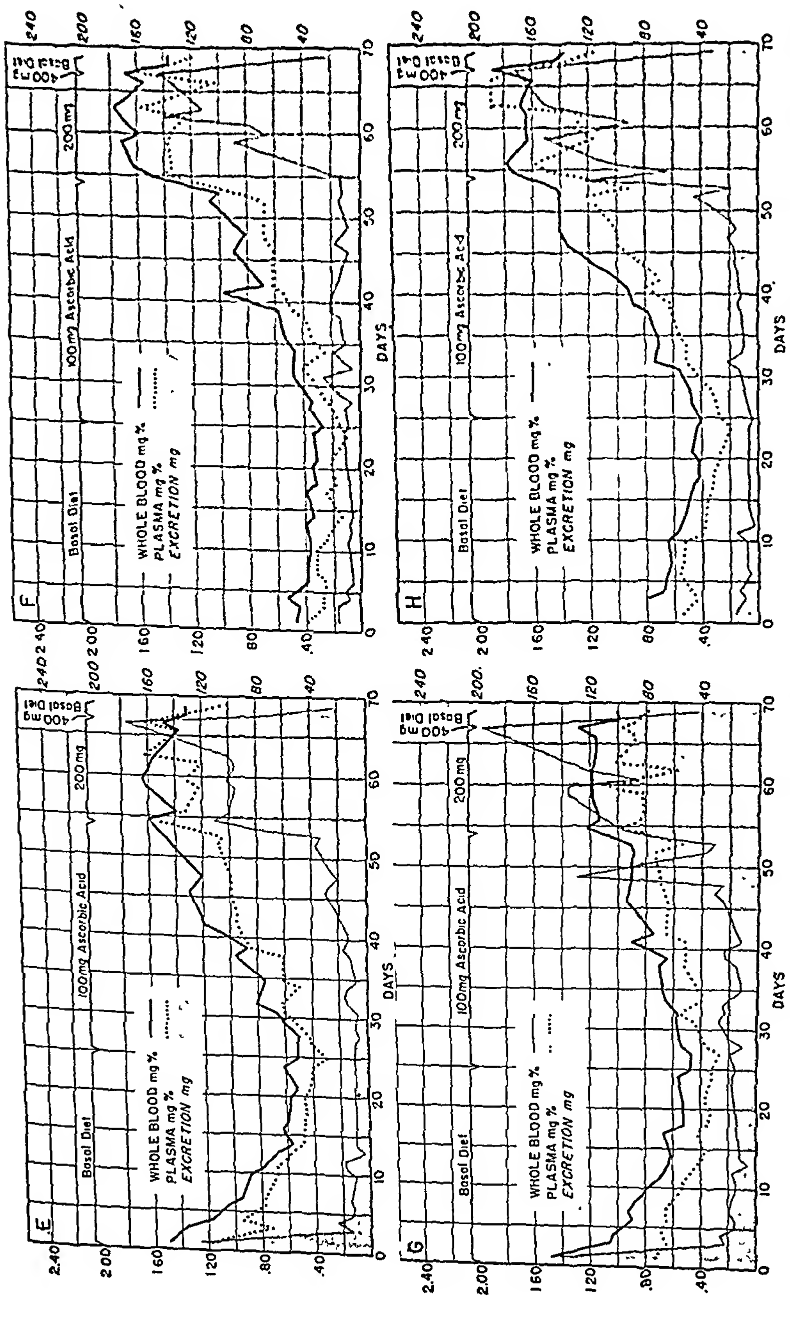

Fig. 2 E, F, G and H refer to subjects 5, 6, 7 and 8, respectively. Left ordinate, scale for plasma and whole blood. Right ordinate, scale for 24-hour urinary excretion.

and of Kyhos et al. ('45), who observed ascorbic acid concentrations of plasma to be greater than those of whole blood only transiently, following absorption of ascorbic acid, when the exchange of the vitamin between serum and cells caused fluctuations in their respective concentrations of it.

It should be noted that large differences between plasma and whole blood ascorbic acid values occurred in only a few instances. The average difference between plasma ascorbic acid values and those of whole blood on the first and third days of depletion was 0.38 mg %, while the average difference for the remainder of the depletion period was 0.15 mg %, showing a tendency for whole blood values to approach those of plasma somewhat more closely after the first days of depletion. There was no consistent trend either toward smaller or toward larger differences between plasma and whole blood ascorbic acid values following the large differences found at the beginning of depletion. The average difference for all samples during both depletion and repletion was 0.18 mg %, while the average difference reported by Lubschez ('45) was 0.14 mg %. The coefficient of correlation among the 283 comparable plasma and whole blood ascorbic acid values obtained during the entire experiment was 0.936, which is highly significant.

Estimates of the error variance, i.e., the variance among values for the same individual on a given day, for whole blood and plasma were very similar, being 0.0102 and 0.0091 respectively (Lucas, '48). In addition, estimates of the variance among subjects with regard to plasma and whole blood values were nearly the same. Thus these data do not indicate a choice between the two values from the point of view of random variabilities.

Determination of the ascorbic acid levels of whole blood, however, has the advantage of including white cells, which were found by Crandon and associates ('40) and by Pijoan and Lozner ('44) to retain an appreciable ascorbic acid value for a considerable time after the vitamin disappeared from the plasma.

The urinary excretion of ascorbic acid, as might be predicted, followed a different pattern from that of the whole blood and plasma values. In contrast to the gradual fall of whole blood and plasma ascorbic acid values, the average 24-hour ascorbic acid excretion of the 8 subjects fell abruptly from 108 mg on the last day of the preliminary period to 14 mg on the third day of the depletion period. Further decreases during the depletion period were small and irregular. Considerable individual variation was observed in the excretion of subjects having similar plasma and whole blood ascorbic acid values. At the beginning of repletion the urinary excretion rose slowly and irregularly at first, tending to show a sudden marked increase, which occurred in two subjects while taking the 100 mg and in the remainder while taking the 200 mg supplement. In those subjects who had shown a large increase in excretion on the 100 mg supplement, increase of the supplement did not cause a corresponding increase in excretion. Similarly, the 400 mg test dose given to subjects 5, 6, 7 and 8, following two weeks when 200 mg of ascorbic acid had been taken daily, was followed by increased retention. This increase in retention when intakes of ascorbic acid are increased has been reported previously by Johnson and Zilva ('34) and by Hamel ('37). The first 24-hour excretion of one-third or more of the intake coincided with plasma values of 0.78 to 1.59 mg % (figs. 1 and 2). When supplements were withdrawn at the end of the repletion period the urinary excretion for the two succeeding days again showed a marked decline.

It may be concluded that data based on the urinary excretion of ascorbic acid must be interpreted with caution. The large fluctuations in excretion values for the same individual on a constant low intake, and the slow and irregular response of these values to large increases in intake, leave serious doubts as to the reliability of a test of the habitual or "resting level" of the urinary excretion of ascorbic acid as a criterion of nutritional status. In the present investigation, however, large increases in excretion in response to an intake of 200 mg

or more of ascorbic acid coincided with plasma levels of 0.8 mg % or above, which is commonly accepted as being indicative of a satisfactory state of nutrition with regard to this vitamin.

CONCLUSIONS

1. It may be concluded that fasting plasma ascorbic acid values are in most instances lower than those of whole blood.
2. There is no level at which plasma ascorbic acid values are consistently higher than those of whole blood and which may be used as a criterion of blood cell saturation.
3. The random variabilities of plasma and of whole blood are very similar.
4. Urinary excretion of ascorbic acid is a poor indication of nutritional status with respect to this vitamin except when very high excretion values, indicating saturation, are obtained.

ACKNOWLEDGMENTS

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EFFECT OF ARSONIC ACID DERIVATIVES IN STIMULATING GROWTH OF CHICKENS

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TWO FIGURES

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Morehouse and Mayfield ('46), in reporting the effect of some aryl arsonic acids on experimental coccidiosis in chickens, stated that 3-nitro, 4-hydroxyphenyl arsonic acid had remarkable growth-stimulating properties for chickens and turkeys when given in the drinking water at 25% of the concentration necessary for coccidiosis control. The data on which this statement was based were not included in the article, but the authors kindly made available to us their unpublished material. The improvement in growth which they obtained in their experiments as the result of administering the abovementioned compound was indeed remarkable. It seemed possible that this compound might be performing the same function as the unknown growth and hatchability factor which experiments in this laboratory have shown to be present in fish meal and in the feces of cows and chickens (Rubin and Bird, '46). Experiments were therefore planned to determine the optimum dietary level of 3-nitro, 4-hydroxyphenyl arsonic acid for stimulating the growth of chickens, to test the effectiveness in this respect of compounds chemically related to it, and to compare its effectiveness in several diets with the effectiveness of concentrates of the unknown factor.

METHODS AND RESULTS

Determination of optimum level

The formulas for the basal diets are given in table 1. Diet A was used in the two experiments designed to determine the optimum level of 3-nitro, 4-hydroxyphenyl arsonic acid for growth and the extent of the growth response that could be achieved at this level. In each experiment individual groups

TABLE 1

Composition of basal diets

INGREDIENTS	DIET A	DIET B	DIET C
	%	%	%
Yellow corn	31.0	38.0	23.0
Barley	10.0	20.0	...
Wheat	20.0	.	...
Alfalfa meal	5.0	3.0	3.0
Soybean meal	30.0	35.0	70.0
Butyl fermentation solubles (250 μg riboflavin per gram)	0.6	0.6	0.6
Steamed bonemeal	1.5	1.5	1.5
Limestone flour	1.0	1.0	1.0
Manganized salt [1]	0.5	0.7	0.7
Iodized salt [2]	0.2		
Vitamin A and D oil [3]	0.2	0.2	0.2
	100.0	100.0	100.0
Nicotinic acid (mg/100 gm)		1.0	1.0

[1] Ninety-six per cent NaCl and 4% $MnSO_4 \cdot 4H_2O$.

[2] NaCl 100 parts, KI 0.3, $Na_2S_2O_3 \cdot 5H_2O$ 0.4, $CaCO_3$ 3.3, cornstarch 6.0.

[3] Four hundred A.O.A.C. units of vitamin D and 2000 I.U. of vitamin A per gram.

were fed diets which contained, respectively, 0, 0.0025, 0.005, and 0.01% of 3-nitro, 4-hydroxyphenyl arsonic acid and in one experiment an additional group received 0.02%. In each experiment one group received 5% of fish meal. Groups of 40 chicks were housed in the separate sections of a long brooder house. Each section contained an electrically heated hover and a steam radiator to provide supplementary heat. Wood shavings were used as litter. The chicks were three-

way crossbreds, being the progeny of New Hampshire males and Rhode Island Red X Barred Rock crossbred females. The dams of the chicks used in experiment 2 received different diets, some of which were adequate and some deficient with respect to the unknown factor required for growth and hatchability. To allow for the expected early mortality, lots of 50 day-old chicks were started on the experiment and the number reduced to 40 when the chicks were one week old by removing the smallest surviving chicks from each lot. The chicks in

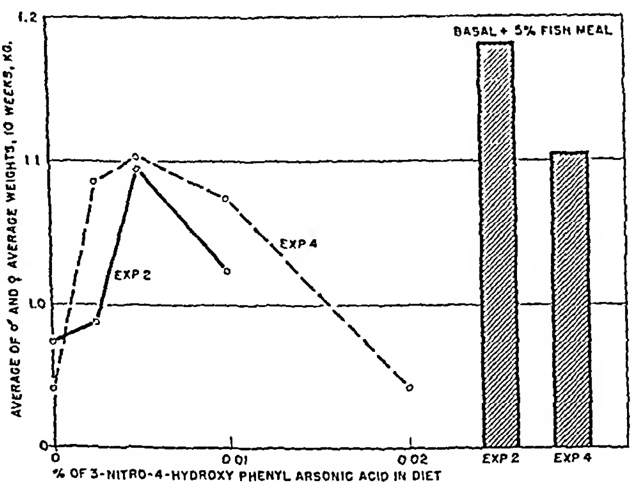

Fig. 1 Average weights of 10-week-old chickens fed 3-nitro, 4-hydroxyphenyl arsonic acid (at various levels) or 5% of fish meal as a supplement to diet A.

experiment 4 were all from dams that received diets adequate with respect to the unknown factor. The chickens were weighed at intervals of two weeks until they were 10 weeks old, at which time the experiments were terminated.

The results are given in figure 1. The optimum level of the arsonic acid derivative was 0.005% of the diet, and the groups fed this amount showed a substantially greater body weight than the basal groups at 10 weeks of age, the differences being 121 gm in experiment 2 and 163 gm in experiment 4. The response due to the arsonic acid derivative was com-

parable to that due to 5% of fish meal in experiment 4, but not in experiment 2.

Testing of related compounds

Diets A and B (table 1) were used in these experiments. The various compounds tested for their effect on growth are

TABLE 2

Average weights at 6 weeks of age of chickens fed various compounds

SUPPLEMENT TO BASAL DIET [1]	AVERAGE BODY WEIGHT AT 6 WEEKS IN EXPERIMENT NUMBER			
	5	6	7	8
	gm	*gm*	*gm*	*gm*
None	245	312	357	366
0.075% of acid ppt. of H_2O extract of cow manure		413	494	424
0.005% 3-nitro, 4-hydroxyphenyl arsonic acid	380	357	411	437
0.000375%-$Na_2HAsO_4 \cdot 7H_2O$	266			. .
0.00075%-$Na_2HAsO_4 \cdot 7H_2O$	262		.	. . .
0.0015%-$Na_2HAsO_4 \cdot 7H_2O$	243		. .	. . .
0.003%-$Na_2HAsO_4 \cdot 7H_2O$	246		. . .	. . .
0.005% phenyl arsonic acid		416 [2]	434	368
0.005% *p*-hydroxyphenyl arsonic acid		373 [3]	425	395
0.005% *p*-chlorophenyl arsonic acid		327		. . .
0.005% *m*-nitrophenyl arsonic acid			398	399
0.005% 3-nitrosalicylic acid			375	365
0.005% *m*-nitrobenzenesulfonic acid			331	360
0.005% sodium *p*-phenolsulfonate			338	320
0.005% salicylic acid			335	369
Least significant difference, odds of 19:1	50	62	64	50

[1] Basal diet A was used in experiment 5 and basal diet B in the other experiments.

[2] Level fed was 0.004 rather than 0.005%.

[3] Level fed was 0.0045 rather than 0.005%.

listed in table 2. In experiment 5, sodium arsenate was compared with 3-nitro, 4-hydroxyphenyl arsonic acid as a supplement to diet A. In the next three experiments diet B, the same diet used in the studies on the unknown growth factor, was used and the diet of the positive control groups was sup-

plemented with 0.075% of the acid precipitate of the water extract of cow manure to supply the unknown factor (Rubin and Bird, '46). It is not believed that the differences between diets A and B are of any significance with respect to these experiments.

The chicks used in experiments 5 and 6 were three-way crossbreds of the same derivation as those described above. The chicks used in experiment 7 were the crossbred progeny of Barred Rock males and Rhode Island Red females. The dams of the chicks used in these three experiments received a diet deficient in the unknown growth factor. Rhode Island Red chicks from hens fed a "complete" diet were used in experiment 8. Each group contained 25 chicks in experiment 5 and 12 in each of the other experiments. The chicks in experiment 5 were fed the experimental diets from hatching time to the age of 6 weeks; those in the other experiments, from the ages of two to 6 weeks. When the chicks were distributed among the experimental groups at two weeks of age, the very light and very heavy chicks were discarded and the groups equalized with respect to average weight. All groups were housed in steel batteries with screen floors.

The results are summarized in table 2. The data were analyzed by the method of Titus and Hammond ('35). The chickens fed 3-nitro, 4-hydroxyphenyl arsonic acid weighed more in all cases than did those fed the basal diet. The differences were statistically significant in experiments 5 and 8 and fell short of statistical significance in experiments 6 and 7. Phenylarsonic acid produced a marked stimulus to growth in experiments 6 and 7 and no stimulus in experiment 8. The chicks fed *p*-hydroxyphenyl arsonic acid were heavier than those fed the basal diet in all three experiments. In one experiment the result was clearly significant; in the other two it fell short, though only by one gm in experiment 6. In two experiments there appeared to be some growth response due to *m*-nitrophenyl arsonic acid but in neither case was the difference statistically significant. Growth was not stimulated by sodium arsenate, *p*-chlorophenyl arsonic acid, 3-nitrosal-

icylic acid, *m*-nitrobenzenesulfonic acid, sodium *p*-phenolsulfonate, or salicylic acid.

Comparative growth response of chickens fed the 3-nitro, 4-hydroxyphenyl arsonic acid, the unknown growth factor and methionine with different diets

As shown in figure 1 and in table 2, 3-nitro, 4 hydroxyphenyl arsonic acid stimulated growth when added to diets deficient in the unknown growth factor. In some experiments it was as effective as a concentrate of this factor; in others it was not.

TABLE 3

Average weights at 6 weeks of age of chickens fed various supplements with a diet containing 70% of soybean meal

GROUP NO.	SUPPLEMENT TO DIET O	AVERAGE WEIGHTS AT 6 WEEKS	
		By groups	By diets
		gm	*gm*
1	None	295	311
2	None	326	.
3	5% dried cow manure	467	453
4	5% dried cow manure	441	.
5	0.4% DL-methionine	360	338
6	0.4% DL-methionine	315	.
7	0.005% 3-nitro, 4-hydroxyphenyl arsonic acid	385	397
8	Same as 7	408	

In an attempt to determine if the two supplements served the same function they were added to a diet containing 35% of raw soybeans rather than processed meal and to a diet containing 70% of processed soybean meal. It has been shown (Rubin and Bird, '47) that the latter diet is very effectively supplemented by the unknown growth factor but not by DL-methionine, and that the unknown factor is less effective in supplementing raw than processed soybean meal.

An experiment was performed in which diet C (table 1), containing 70% of soybean meal, was fed to 8 lots of chickens

with the supplements listed in table 3. The chickens were three-way crossbreds of the same derivation as those described above. The groups were housed in the separate pens of a brooder house with shavings for litter, and were fed the experimental diets from the ages of one to 6 weeks.

The results summarized in table 3 show that the arsonic acid derivative was effective when fed with 70% of soybean meal but less effective than was 5% of dried cow manure,

TABLE 4

Effect of type of soybean product and of supplements on average weights of chickens at 6 weeks of age

MODIFICATION OF DIET B		AVERAGE BODY WEIGHTS OF CHICKENS AT 6 WEEKS IN EXPERIMENT NUMBER		
Soybean product	Supplement [1]	9	10	11
		gm	*gm*	*gm*
Raw soybeans	None	221	318	289
Raw soybeans	A.A.	289	375	327
Raw soybeans	A.P.	324	390	314
Raw soybeans	M.	347	422	
Raw soybeans	A.A. + A.P.	387	412	368
Raw soybeans	A.A. + M.	398	444	389
Raw soybeans	A.P. + M.	370	434	401
Raw soybeans	A.A. + A.P. + M.	411	462	411
Heated soybean meal	A.P.			449

[1] A.A. = 3-nitro, 4-hydroxyphenyl arsonic acid; 0.005% in experiments 9 and 10, 0.01% in experiment 11.

A.P. = acid precipitate of water extract of cow manure; 0.075% in experiments 9 and 10; 0.15% in experiment 11.

M. = DL-methionine; 0.4%.

which supplied the unknown growth factor. As reported previously DL-methionine was ineffective in promoting growth.

Since varying the dietary level of soybean meal did not lead to any definite conclusion as to whether the effects of the growth factor and the arsonic acid were identical, three experiments were performed in which these supplements and methionine were fed alone and in various combinations in a diet containing 35% of raw soybeans instead of heated soybean

meal. Groups of 12 crossbred chicks (Barred Rock X Rhode Island Red) were used in all three experiments. During the first two weeks of life they were fed diet B containing heated soybean meal without supplement. At two weeks of age they were divided into groups according to the procedure described above. From that time until they were 6 weeks of age they were fed the experimental diets, which were modifications of diet B. The nature of the dietary modifications and the average weights of the chickens are summarized in table 4.

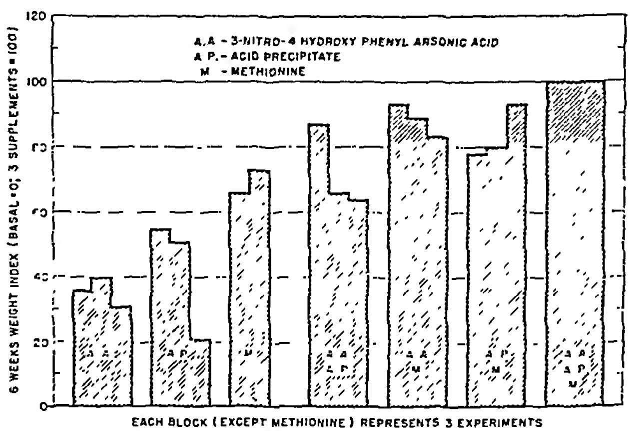

Fig. 2 Effects of 3-nitro, 4-hydroxyphenyl arsonic acid, the acid precipitate of a water extract of cow manure, and DL-methionine, fed separately and in various combinations, on the growth of chickens up to the 6th week.

To facilitate comparison of the three experiments the same data are summarized in figure 2. In each experiment the weight of the group receiving all three supplements was taken as 100 and the weight of the basal group as 0, and a value was calculated for each of the other groups to show its position between the basal group and the group receiving the supplements. It was obvious (1) that the methionine was the most effective of the single supplements; (2) that any pair of supplements was superior to any supplement alone (except that in experiment 10 methionine alone was superior to the other two supplements in combination); and (3) that the three

supplements together were superior to any pair. These results indicate that the three materials were mutually supplementary.

As shown in the footnote to table 4, the levels of the arsonic acid derivative and the acid precipitate were doubled in experiment 11. This was done to make sure that the apparent effect of the supplements observed in experiments 9 and 10 was not simply additive. The higher levels of the single supplements were not more effective in promoting growth than the lower levels. This result rules out the possibility of an additive effect.

A positive control group which received commercial heated soybean meal supplemented with the unknown factor was included in experiment 11. As shown in table 4, the diet containing raw soybeans plus all three supplements was still slightly inferior to the positive control diet.

DISCUSSION

The results of these studies confirm the report of Morehouse and Mayfield ('46) that 3-nitro, 4-hydroxyphenyl arsonic acid stimulates the growth of chickens when fed with certain diets. The optimum level of this compound for stimulating growth was 0.005% of the diet. The chickens fed 0.01% were definitely inferior to those fed 0.005% and those receiving 0.02% were no better than the basal group, showing that any attempt to make practical use of this compound would necessitate very careful regulation of the level fed.

Of the compounds tested only phenyl arsonic acid and its nitro- and hydroxy-derivatives were effective in promoting growth. The presence of the nitro- and hydroxy-groups was not essential. On the contrary, their presence may have resulted in reduced effectiveness although this was not definitely established by these experiments.

Tangl ('42) reported that the growth of chickens was stimulated by feeding cupri-sulfarsenite at a level supplying 0.035 mg of arsenic per bird per day. The composition of the basal diet was not stated. If there is a common denomina-

tor that accounts for Tangl's results and those reported in this paper, it will necessarily be a simple inorganic arsenic compound, especially since the compound fed by Tangl was more effective per unit weight of arsenic than the arsonic acid derivatives. The 3-nitro, 4-hydroxyphenyl arsonic acid fed as 0.005% of the diet supplied approximately 0.5 mg of arsenic per chicken per day. The 4 levels of sodium arsenate (table 2) supplied, respectively, 0.03, 0.06, 0.12 and 0.24 mg of arsenic per chicken per day.

Although sodium arsenate was not effective, it is possible that inorganic arsenic may exert a stimulating effect when liberated slowly within the organism. The metabolism of the arsenic compounds used in these experiments was not investigated, but it was found by Crawford and Levvy ('47) that after intravenous injection of phenyl arsonic acid into rabbits 90% of the urine arsenic was in the form of phenyl arsonic acid and 10% in the form of phenyl arsenious acid; no arsenious or arsenic acid was found. Apparently phenyl arsonic acid does not readily liberate inorganic arsenic compounds in the rabbit.

According to Gordon and Quastel ('48), organic trivalent arsenoxides combine reversibly with the thiol groups of enzymes, thus acting as inhibitors to the thiol enzymes. Although pentavalent organic arsenic compounds did not directly affect such enzymes, they were reduced by thiol compounds to trivalent arsenoxides which did combine with the enzymes. Enzyme inhibition is not a likely explanation for a growth-stimulating effect, but the possibility that this effect may involve reaction with some biologically important thiol compound is an interesting one.

The experiments with the diet containing raw soybeans indicate that the effect of the arsonic acid derivatives is distinct from the effects of the unknown growth factor and methionine, since these three materials supplemented each other. Since the arsonic acid derivative was more effective when fed with heated soybean meal than when fed with raw soy-

beans, its effect is not believed to be related to the trypsin inhibitor found in raw soybeans.

SUMMARY

The growth of chickens fed diets high in soybean meal and deficient in the unknown dietary factor found in fish meal and in cow manure was improved by the addition to the diet of 0.005% of 3-nitro, 4-hydroxyphenyl arsonic acid. The effect of higher or lower levels was less favorable. This compound did not function as a substitute either for the unknown factor or for methionine. On the contrary, with diets containing raw soybeans the arsonic acid derivative, the unknown factor, and methionine were mutually supplementary. The arsonic acid derivative was effective when fed with 35% or with 70% of commercially heated soybean meal, but less effective than the unknown factor. Other compounds comparable in their activity to 3-nitro, 4-hydroxyphenyl arsonic acid were phenyl arsonic acid and *p*-hydroxyphenyl arsonic acid. *M*-nitrophenyl arsonic acid also showed some growth-promoting activity. The following were tested and found inactive in this respect: *p*-chlorophenyl arsonic acid, sodium arsenate, *m*-nitrobenzenesulfonic acid, salicylic acid, 3-nitrosalicylic acid, and sodium *p*-phenolsulfonate.

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ADDENDUM

After this paper was submitted, evidence was obtained which indicated that the "unknown factor" mentioned here is vitamin B_{12}. (Lillie, R. J., C. A. Denton, and H. R. Bird, '48. Relation of vitamin B_{12} to the growth factor present in cow manure. J. Biol. Chem., *176:* 1477.)

PANTOTHENIC ACID AND CONJUGATION REACTIONS IN VIVO

I. THE EFFECT OF PANTOTHENIC ACID AND THIAMINE DEFICIENCIES ON THE ABILITY OF THE RAT TO ACETYLATE SULFANILAMIDE [1]

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ONE FIGURE

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The finding that pantothenic acid is a constituent of Coenzyme A (Lipmann et al., '47; Kaplan and Lipmann, '47) which functions in the acetylation of aromatic amines and choline *in vitro* led naturally to a study of the *in vivo* role of this vitamin in various acetylation reactions. Martin and Rennebaum ('43) had previously reported, on the basis of observations of blood levels, that pantothenic acid- and pyridoxine-deficient rats acetylated sulfanilamide at a normal rate, whereas thiamine- and riboflavin-deficient rats were defective in this respect.

While the present study was underway Riggs and Hegsted ('48) presented evidence for an *in vivo* role of pantothenic acid in the acetylation of *p*-aminobenzoic acid. This report on the acetylation of sulfanilamide confirms their observations and presents additional evidence on this subject.

[1] This work was supported by a grant from the National Vitamin Foundation. Preliminary studies were carried out with the assistance of a grant (RG 312c) from the U. S. Public Health Service.

METHODS

Pantothenic acid deficiency was induced by feeding a diet (Diet III-P) with the following percentage composition: Casein (vitamin-free) 20; cerelose 66; corn oil 10; salts (GBI salt mixture no. 2) 4; and with the following vitamins per 100 gm of the above basal mixture: percomorph oil, three drops; thiamine, 1 mg; riboflavin, 1 mg; pyridoxine, 1 mg; niacin, 2 mg; folic acid (Folvite), 0.20 mg; biotin, 0.002 mg; and choline, 100 mg. The control diet (Diet III) contained in addition 5 mg of calcium pantothenate per 100 gm of basal diet. The thiamine-deficient diet was similar to diet III except for the omission of thiamine.

Urine was collected over a 22-hour period from individual rats in metabolism cages. Sulfanilamide was injected intraperitoneally as an 0.8% water solution and analysis for the free and acetylated forms was performed by the method of Bratton and Marshall ('39). The percentage of the total excreted sulfanilamide which was acetylated was calculated and served as the basis for comparison in the following experiments.

EXPERIMENTAL PROCEDURES AND RESULTS

1. *Sulfanilamide dosage and acetylation rate*

Without prior knowledge of the sensitivity of the acetylation mechanisms in relation to dosage and to possible effects of pantothenic acid deficiency, it was important to determine the optimum conditions for estimating the effects of the deficiency. The administration of too much sulfanilamide would flood the organism with the aromatic amine, so that the percentage acetylated by the rat would be low; too little of the drug might not tax the acetylation mechanism to the point where the effect of a deficiency would be manifest.

The percentage acetylation by both control and pantothenic acid-deficient rats was determined when different amounts of sulfanilamide were injected at varying intervals. The acetylation with two, three or 4 hourly injections of 16 mg each of

sulfanilamide as an 0.8% solution did not differ appreciably, indicating that the acetylation mechanism was not overtaxed. With 5 hourly injections there was a definite decrease in acetylation by both the deficient and the control animals. Nevertheless consistent results were obtained, but at lower percentage levels. While any one of these dosage procedures would be useful in testing acetylation, three hourly injections seem most suitable for two reasons, namely (a) insuring the excretion of an adequate amount of sulfanilamide for analytical purposes without the danger of flooding the organism unduly, and (b) avoiding an unnecessarily large number of injections.

It was noted that normal young male rats weighing about 75 gm or less acetylated much smaller percentages than when older and heavier. This makes it impossible to get a satisfactory base line before placing very young rats on a deficient diet.

2. *Pantothenic acid deficiency and acetylation*

Several experiments were carried out with male rats to determine the acetylation ability with progressive deficiency and recovery.

In figure 1 is shown the course of events when 9 male rats, initially weighing about 70 gm, were fed the deficient diet, while each of 7 paired litter-mate rats were fed a control diet in amounts similar to or below that of the deficient litter mate. All of these rats received hourly intraperitoneal injections of 2 ml of an 0.8% sulfanilamide solution for the first 5 hours of urine collection. Within three weeks significant differences in the percentage of sulfanilamide acetylated were noted between control and deficient animals.

The ability of the deficient rats to recover their power to acetylate following calcium pantothenate administration is also shown in figure 1. On the day indicated by the first arrow, three of the 8 remaining deficient rats were injected with 250 μg calcium pantothenate and the acetylation test carried out the same day. Thereafter these three animals received

the control diet containing 5 mg of calcium pantothenate per 100 gm of diet. A similar procedure was followed with another three deficient rats on the 65th day. The pantothenate injections had no immediate effect but maintenance on the control diet brought acetylation levels back to normal.

In another experiment pantothenic acid deficiency was induced in somewhat older male animals and maintained for approximately twice as long. The control animals were maintained at approximately the same weights as the deficient

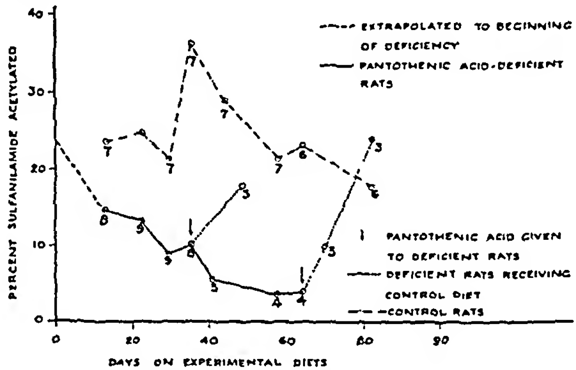

Fig. 1 Effect of pantothenic acid deficiency in rats on acetylation of sulfanilamide. Numbers on the curves refer to the number of animals on which averages were taken. The first analyses were made after 8 days on the experimental diets. Five hourly injections, each of 16 mg sulfanilamide, were made at the beginning of each 22-hour urine collection.

rats by restriction of food intake. Data on acetylation percentages of these control and deficient animals are given in table 1. The data in the first column (—4 days) were obtained to check the uniformity of acetylation of all test animals while on the control diet, and were obtained with two injections (at zero and two hours) of 16 mg sulfanilamide each. All other data were obtained with three hourly injections at zero, one, and two hours of the urinary collection period; 24, 16, and 16 mg of sulfanilamide being given, respectively. The acetylation levels differ from those in figure 1 primarily because of the different amounts of sulfanilamide injected in the two series.

Both groups had similar levels of acetylation in the pre-deficiency period (—4 days), the mean differences between them being insignificant (P = .08 by the "t" test). As the deficiency progressed, marked decreases occurred in the

TABLE 1

Acetylation of sulfanilamide excreted by pantothenic acid-deficient and control rats. All values are in percentages.

DEFICIENT RAT NO.	DAY OF EXPERIMENT							
	− 4[1]	+ 70	+ 79	+ 87	+ 94	+ 98[4]	+ 105[5]	+ 115[6]
40D	40.0	15.3	16.8[2]	14.1	16.2	19.0	18.3	29.5
41D	34.0	14.5	15.9[2]	15.3	15.4	12.3	13.1	26.4
42D	36.4	16.3	15.7[2]	10.0	12.3	9.5	10.4	25.2
43D	36.0	23.6	18.9[3]	21.0	22.5	17.0	18.2	
44D	40.3	16.2	14.1[3]	16.1	16.0	16.2	15.3	28.6
45D	44.6	25.0	21.5[3]		19.1	16.5	18.1	29.8
46D	28.4	15.2	12.4[3]	10.8	14.2	13.4	14.5	27.6
48D	38.2	18.2	14.9[2]	11.5	11.2	11.7	13.0	23.6
Average	37.2	18.0	16.3	14.1	15.9	14.5	15.1	27.2
CONTROL RAT NO.								
40C	48.5	39.6	32.5[2]	32.8	32.2	28.9	31.6	29.0
41C	47.3	35.4	31.7[2]	29.4	28.6	31.2	30.4	29.6
42C	38.2	35.9	32.1[2]	34.2	33.4	31.7	33.2	29.0
43C	39.3	31.8	33.1[3]	27.9	50.0	32.4	32.2	
44C	37.4	34.0	29.4[3]	25.8	28.7	29.4	28.4	33.0
45C	39.3	29.7	37.0[3]	30.2	30.1		29.1	33.9
46C	41.4	33.0	29.2[3]	35.6	32.2	28.6	27.8	27.7
47C	35.2	35.6	32.1[2]	29.0	26.5	30.5	34.3	
48C	33.8	31.2	28.8[2]		33.0	24.1	29.6	31.8
Average	40.0	34.0	31.8	30.6	32.7	29.6	30.7	30.6

[1] On day — 4 all rats were tested for acetylation ability while on control diets, receiving only two injections of sulfanilamide (16 mg each at zero and two hours). The data in the following columns were obtained in acetylation tests with three injections of 16 mg of sulfanilamide at zero, one, and two hours.

[2] Rats received three successive daily injections of 0.2 ml of adrenal cortex extract (Upjohn) before acetylation test.

[3] Rats received one injection of 0.2 ml of adrenal cortex extract (Upjohn) several hours before acetylation test.

[4] Rats received an injection of 100 μg calcium pantothenate several hours before acetylation test.

[5] Rats received 4 injections of 100 μg calcium pantothenate each, spaced evenly during the week preceding acetylation test.

[6] Rats on control diet for one week preceding acetylation test.

acetylation percentages of the deficient group. After 70 days of deficiency, the average of the deficient animals was 53% that of the controls; this difference is highly significant ($P < .001$). As the deficiency period continued there was not much change in the acetylation percentage.

Corresponding to the results obtained in the initial experiment, 4 injections of calcium pantothenate (100 μg each) once every other day for one week had no appreciable effect on the deficient rats (105th day), but ingestion of the control diet for one week caused a marked increase in acetylation percentage (115th day).

None of the deficient animals exhibited severe symptoms; weight loss was gradual but porphyrin-like incrustations about the face were common. There was no close correlation between the extent of weight loss and the acetylation percentage.

Because of the relation of pantothenic acid to the structural integrity and function of the adrenal gland (Daft et al., '40; Ralli and Graef, '45; Deane and McKibbin, '46), the effect of adrenal cortical extract on acetylation was tested. Ten deficient and 10 control rats received subcutaneous injections of 0.2 ml extract of the adrenal cortex [2] on three successive days. The usual sulfonamide test and urine collection started 4 hours after the third administration. Another 4 deficient and 4 control rats received a single injection (0.2 ml) of the hormone preparation 4 hours before the beginning of the acetylation test. The particular batch of extract used was assayed in our laboratory for unbound pantothenic acid by the method of Skeggs and Wright ('44) and found to contain an insignificant amount (0.024 μg/ml). As is indicated in table 1 the extract had no significant effect on acetylation (79th day).

3. *Thiamine deficiency and acetylation*

Despite the food restriction of the control rats in the experiments described above, their physical appearance was much superior to that of the deficient animals. The question

[2] Upjohn's.

presented itself of whether the decreased acetylation of the deficient animals was due to general debility rather than to a specific lack of pantothenic acid. To help answer this question, and at the same time to provide a comparison with the findings of Martin and Rennebaum ('43), acetylation tests were performed on thiamine-deficient rats. In table 2 are given the averages of the percentage acetylation of sulfanilamide (5 hourly injections of 16 mg each) by thiamine-deficient male rats at various intervals and of their litter-mate controls kept

TABLE 2

Effect of thiamine deficiency on ability of the rat to acetylate sulfanilamide

TIME ON EXPERIMENTAL DIETS	AMOUNT OF SULFANILAMIDE ACETYLATED BY	
	Deficient rats [1]	Control rats [1]
	%	%
Control period	17.8	16.6
9 days	21.2	24.1
15 days	24.1	16.4
22 days	16.7	25.5
33 days	19.0	20.2
43 days	25.0	20.4
Average	20.6	20.5

[1] Average of 4 to 6 rats. All animals received intraperitoneal injections of 16 mg of sulfanilamide (0.8% solution) at zero, one, two, three and 4 hours of the 22-hour urine collection period.

at approximately the same weights by restricted feeding. During the last two weeks of the experiment it was necessary to give occasional injections of small amounts of thiamine to the deficient animals to keep them alive. Despite the acute deficiency, there was no significant difference in acetylation percentages between the two groups. This experiment was repeated with smaller amounts of sulfanilamide (24, 16, and 16 mg at zero, one, and two hours) and again no significant differences were noted between control and deficient male rats.

DISCUSSION

The findings reported here on the effect of pantothenic acid deficiency on the ability of rats to acetylate sulfanilamide confirm the *in vitro* studies of Lipmann et al. ('47) on the acetylation of sulfanilamide and the *in vivo* studies of Riggs and Hegsted ('48) on the decreased acetylation of *p*-aminobenzoic acid in pantothenic-deficient rats.

Our observation that thiamine deficiency exerted no influence on the ability of male rats to acetylate sulfanilamide does not support the finding of Martin and Rennebaum ('43) that thiamine deficiency in rats decreased the proportion of acetylated sulfanilamide in the blood. Nor is it in accord with the reports of Kinnunen ('46) and Suomalainen and Kinnunen ('46) that rabbits on a diet composed chiefly of oats and hay excreted a larger proportion of ingested sulfapyridine in the acetylated form following thiamine administration. Incidentally, no evidence was given by these workers that the rabbits on the oats-hay diet were deficient.

Failure to obtain an immediate acetylation response to injected calcium pantothenate (100 or 250 μg) is in keeping with the results of Riggs and Hegsted ('48), who found that while 1 mg of the vitamin restored acetylation of *p*-aminobenzoic acid to normal, 500 μg and 200 μg resulted in inconsistent and incomplete increases. It is noteworthy, however, that 4 injections of 100 μg each over a period of a week did not cause any significant response in our rats.

Quantitative data on the relationships among pantothenic acid intake, tissue Coenzyme A content, and acetylation rate would appear desirable for a variety of reasons, including the possibility of utilizing the acetylation reaction as a simple biochemical assessment of pantothenic acid status.

SUMMARY

1. Rats maintained on a pantothenic acid-deficient diet excreted much smaller percentages of test doses of sulfanilamide in the acetylated form than did control animals.

2. Adrenal cortical extract had no influence on the reduced ability of pantothenic acid-deficient rats to acetylate sulfanilamide.

3. Thiamine deficiency did not affect the ability of male rats to acetylate sulfanilamide as indicated by urinary excretion values.

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THE EFFECT OF THE LEVEL OF DIETARY CALCIUM AND MAGNESIUM ON THE DIGESTIBILITY OF FATTY ACIDS, SIMPLE TRIGLYCERIDES, AND SOME NATURAL AND HYDROGENATED FATS [1, 2, 3]

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ONE FIGURE

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Most natural fats and oils melting below 50°C. have long been recognized as being almost completely digestible. In a summary of previously reported tests on man, Deuel ('48) has noted that of 34 vegetable fats melting below 50°C., only two had coefficients of digestibility below 95. These were avocado oil (87.8%) and tea seed oil (91.2%). In the case of animal fats, 18 different varieties melting under 50°C. were found to be digestible to the extent of 93% or more in man.

There are a number of factors which alter the digestibility of fats. The most important of these is the melting point. Fats

[1] These data are from a thesis presented by Amber L. S. Cheng to the Graduate School of the University of Southern California in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The authors wish to express their appreciation for the use of the facilities of the Hancock Foundation.

[2] Aided by a grant from Swift and Company.

[3] Paper no. 188 from the Department of Biochemistry and Nutrition, University of Southern California.

melting above the critical level of 50°C. have been shown to have considerably reduced digestibilities. Thus, in man, mutton fat (m.p., 50°C.) has been reported to have a coefficient of digestibility of 88 (Langworthy and Holmes, '15), oleostearin (m.p., 50°C.) of 80.1 (Holmes, '19), and deer fat (m.p., 51.4°C.) of 81.7 (Deuel and Holmes, '22). Moreover, straight hydrogenated and blended hydrogenated vegetable oils melting above 50° have been found by Deuel and Holmes ('21, '22) to have lowered coefficients of digestibility. These workers ('21) have suggested that an inverse relationship exists between the absorbability of fats and their melting points.

However, Lyman ('17) suggested that the melting point is not the only factor in determining digestibility but that the fatty acid makeup of the ester is also an important consideration. He based his argument on the fact that tripalmitin, which has a m.p. of 56°C., is utilized to the extent of 95% in the dog, while Arnschink (1890) had earlier reported coefficients of digestibility of 9 to 14 for tristearin (m.p., 71°C.) in the same species. Hoagland and Snider ('43) have obtained similar differences in tests on rats, the coefficients of digestibility being 84 to 82 and 8 to 6 for tripalmitin and tristearin, respectively, when the triglycerides constituted 5 or 10% of an olive oil solution which in turn was fed at a 5% level. Mattil and Higgins ('45) have also demonstrated that some variation in digestibility obtains when comparable fatty acid mixtures are administered as components of a mixed triglyceride, as contrasted with their administration as mixtures of proportionate amounts of simple triglycerides. Thus, the average digestibility of distearomono-olein when fed at a 15% level was 66, while the coefficient of digestibility of a mixture of 10% tristearin and 5% triolein was found to be only 40.

Another factor of importance in determining the digestibility of high melting triglycerides is the nature of the other fat components. When tristearin is dissolved in olive oil (Hoagland and Snider, '43) or is present as a component of natural fats so that the melting point of the mixture is

below 50°C., a high digestibility obtains because of the aid of the solvent action of such fats with lower melting points as triolein. The presence of such emulsifying agents as lecithin also increases the utilization of fats with high melting points (Augur et al., '47).

Moreover, there is some evidence that the species of animal plays a role as a factor in digestibility. Guinea pigs have been shown by McCay and Paul ('38) to have lower capacities for absorption, not only of hydrogenated fats but also of such common vegetable oils as corn oil, in comparison with man. On the other hand, such other herbivora as the rabbit and the sheep were found to digest common fats as well as man (Paul and McCay, '42–'43). An interesting difference between these species and man is the ready digestibility of castor oil in the former group. Although the rat and dog in general have responses to fat comparable to man's, it has recently been shown that considerable amounts of rapeseed oil escape absorption in the rat (Deuel, Cheng and Morehouse, '48) in contrast to its complete digestibility in man (Holmes, '18). The high digestibility of rapeseed oil in man has recently been confirmed in this laboratory.[4]

One important factor, which causes an alteration in fat absorption and which has been investigated only to a slight extent, is the effect of the presence of dietary calcium and magnesium. Givens and Mendel ('17) found that when the absorption of fats or fatty acids was poor, the excretion of fecal calcium was increased. In fact, calcium storage was decreased under such conditions even when the calcium intake was at a high level. Bosworth et al. ('18) concluded that the presence of ionized calcium in the intestine determines the extent of soap formation, although the amount ultimately excreted in the feces depends on the solubility of the soap formed. It was suggested that calcium oleate is more readily soluble in bile than calcium palmitate or calcium stearate; hence, it disappears from the intestine in larger quantities

[4] Unpublished experiments of H. J. Deuel, Jr., R. M. Johnson, C. M. Calbert and B. Thomas.

even in the presence of an excess of calcium. Boyd, Crum and Lyman ('32) reached similar conclusions. When a diet containing a relatively high calcium intake (60–70 mg per day) was fed to rats, the utilization of oleate, palmitate, and stearate soaps was found to be 90, 38, and 25%, respectively; on the other hand, when a relatively low calcium diet was employed (13.5–31.4 mg per day), the absorption of palmitate and stearate was increased to 65 and 45%, respectively. MacDougall ('38), on the other hand, demonstrated an antirachitic effect of lard or olive oil in a low calcium diet; this was explained as being due to an improved absorption of calcium as the oleate; in the absence of fat it was largely lost from the intestine in combination with phytin.

The present experiments were designed to determine the effect of the inclusion of calcium and magnesium in the diet on the excretion of soaps on diets relatively rich in fats. Studies have been made not only with some natural fats but also with a series of simple synthetic triglycerides and also several fatty acids.

EXPERIMENTAL AND RESULTS

The experimental procedure was similar to that used by Augur et al. ('47). Adult female rats weighing between 145 and 185 gm were fed ad libitum, over 7- to 10-day periods, diets containing the fats. In all cases a preliminary orientation period of two days was allowed, followed by an interval during which food consumption was measured and the feces collected for analysis. This latter period was 8 days in length where normal food fats were fed but was shortened to 5 or 6 days where animals were fed diets containing the synthetic triglycerides. Total glycerides and fatty acids were determined in the stools by diethyl ether extraction of the dried stool. Acidification of the fecal residue and a second extraction with ether yielded the portions present as soaps. The composition of the diets used is included in table 1.

Calculations for digestibility were made in the usual manner *after correction for metabolic fat* (Augur et al., '47). The

figures for metabolic fat were determined in tests where the rats had a similar diet but in which the total fat had been replaced by carbohydrate. The data giving the results of these experiments are summarized in table 2.

TABLE 1

Composition of the various diets used

INGREDIENT	DIET A	B	C	D	E	F
Commercial casein (%)	18	18	18	18	18	18
Glucose (cerelose) (%)	59	61	61	61	74	76
Fat (%)	15	15	15	15	0	0
Yeast (Anheuser-Busch, G) (%)	1	1	1	1	1	1
Osborne-Mendel salt mixture (%)	7	5[1]	5[2]	5[3]	7	5
Ca content (mg/gm)	6.1	0	2.5	1.17	6.1	0
Mg content (mg/gm)	0.9	0	0.35	0.16	0.9	0

[1] Salts containing calcium and magnesium were omitted from the Osborne-Mendel salt mixture.

[2] Salts containing calcium and magnesium were added in half the amounts prescribed in the Osborne-Mendel salt mixture.

[3] Salts containing calcium and magnesium were added in one-quarter the amounts prescribed in the Osborne-Mendel salt mixture.

TABLE 2

Summary of control tests to determine metabolic fat of rats fed fat-free diets containing normal Ca and Mg content (diet E) and low in Ca and Mg (diet F)

DATA SUMMARIZED	DIET E (Fat-free containing 7% O.M.[1] salt mixture)	DIET F (Fat-free-containing 5% O.M.[1] salt mixture with Ca and Mg omitted)
Number of rats	10	10
Average weight of rats (gm)	170	166
Average gain or loss (gm)	—4	—1
Average total weight of stools (gm)	3.83	1.53
Average fat excreted		
As neutral fat and fatty acid (mg)	119	149
As soaps (mg)	122	72
Total (mg)	241	221
Average fat/gm stool (mg)	65	148

[1] Osborne-Mendel.

TABLE 3

Summary of digestibility studies on some natural and hydrogenated fats fed with salts of calcium and magnesium (diets A) or without salts of these metals (diet B)

DATA SUMMARIZED	FAT FED											
	Crude rapeseed oil		Refined rapeseed oil		Bland lard		Hydrogenated lard		Blended lard [1]		Hydrogenated lard	
Melting point (°C.)	Liquid		Liquid		47.8		55.4		55.2		61.0	
Diet	A	B	A	B	A	B	A	B	A	B	A	B
Number of rats	10	5	10	5	5	5	5	5	5	5	5	5
Average weight of rats (gm)	175	175	160	165	167	168	177	172	177	168	174	169
Average gain or loss (gm)	+ 4	— 4	— 1	+ 1	+ 2	+ 2	0	— 3	— 1	— 1	— 5	— 1
Average fat ingested (gm)	12.1	10.4	7.8	10.8	13.3	12.2	11.8	9.2	10.7	9.1	11.0	10.6
Average weight of stools (gm)	6.47	2.50	3.42	1.93	5.74	2.73	8.00	3.80	7.00	3.60	12.1	9.9
Average fat excreted												
As neutral fat and fatty acid (gm)	1.07	0.66	0.65	0.57	0.25	0.39	0.51	1.17	1.06	1.33	3.78	5.98
As soaps (gm)	2.12	0.51	0.99	0.39	1.16	0.54	4.67	1.40	2.97	1.16	6.03	1.99
Total (corrected) (gm)	2.77	0.81	1.42	0.66	1.03	0.51	4.66	2.01	3.57	1.75	9.03	6.50
Coefficient of digestibility [2]	77.2 ± 1.6	92.3 ± 1.4	82.5 ± 1.4	93.9 ± 0.6	92.4 ± 0.9	95.8 ± 0.6	58.0 ± 2.4	77.9 ± 4.8	66.2 ± 1.8	80.0 ± 1.9	17.3 ± 3.0	38.0 ± 3.6

[1] A mixture of one-third hydrogenated lard (m.p., 61°C.) and two-thirds prime steam lard (m.p., 37°C.).

[2] Including the standard error of the mean calculated by the formula $\sqrt{\frac{d^2}{n}}/\sqrt{n}$ where "d" is the deviation from the mean and "n" is the number of observations.

TABLE 1

Summary of digestibility studies on some synthetic simple triglycerides fed with calcium and magnesium salts (diets A, C, D) or without salts of these metals (diet B)

DATA SUMMARIZED	FAT FED									
	Trilaurin				Trimyristin		Tripalmitin		Tristearin	
Melting point (°C.)	49				56		66.5		70	
Diet	A	C	D	B	A	B	A	B	A	B
Number of rats	6	5	5	5	5	5	5	5	5	5
Average weight of rats (gm)	170	161	172	168	183	168	173	163	161	169
Average gain or loss (gm)	—4	+ 8	—5	0	+ 5	+ 2	— 2	— 1	0	— 2
Average fat ingested (gm)	8.9	7.6	10.0	8.8	12.8	9.7	9.1	9.8	9.5	9.0
Average weight of stools (gm)	4.80	1.98	2.47	1.55	10.53	4.43	11.67	10.39	12.11	10.56
Average fat excreted										
As neutral fat and fatty acids (gm)	0.25	0.23	0.38	0.16	1.12	1.49	6.10	7.51	7.50	7.97
As soap (gm)	2.72	0.83	0.79	0.29	7.16	1.33	2.27	1.27	1.90	0.88
Total (corrected) (gm)	2.61	0.94	1.01	0.22	7.90	2.26	7.92	7.25	8.61	7.28
Coefficient of digestibility[1]	70.5	87.2	89.5	97.3	37.7	76.6	12.8	27.9	10.6	18.9
	± 1.7	± 4.0	± 0.8	± 3.7	± 1.4	± 8.1	± 1.2	± 1.5	± 2.9	± 2.4

[1] Including the standard error of the mean calculated as described in table 3, footnote 2.

TABLE 5

Summary of digestibility studies on palmitic acid, stearic acid and monostearin fed with calcium and magnesium salts (diet A) or without salts of these metals (diet B) or as soaps (diet G)

DATA SUMMARIZED	FAT FED							
	Palmitic acid			Stearic acid			Monostearin	
Melting point (°C.)	63			69			59.9	
Diet	A	B	G[1]	A	B	H[2]	A	B
Number of rats	5	5	5	5	5	4	5	5
Average weight of rats (gm)	160	162	172	165	158	173	158	145
Average gain or loss (gm)	+1	−1	−13	−3	0	−5	−10	−5
Average fat ingested (gm)	9.7	9.1	9.4	9.8	9.6	9.6	10.1	9.5
Average weight of stools (gm)	10.4	8.9	9.46	11.8	9.80	10.3	12.1	7.37
Average fat excreted								
As neutral fat and fatty acids (gm)	3.89	4.98	1.08	3.99	6.92	1.30	3.22	4.00
As soaps (gm)	4.60	2.29	7.43	5.53	2.78	8.09	5.55	1.84
Total (corrected) (gm)	7.81	5.87	7.89	8.45	8.15	8.73	8.06	4.75
Coefficient of digestibility[3]	19.8	35.6	16.7	14.4	15.8	9.5	20.7	47.4
	± 3.4	± 1.9	± 4.0	± 3.1	± 2.9	± 0.6	± 2.3	± 6.7

[1] Contains 10.00 gm of calcium palmitate and 5.68 gm of palmitic acid per 100 gm food (equivalent to 14.95 gm of palmitic acid and 76 mg of calcium).

[2] Contains 10.47 gm of calcium stearate and 5.21 gm of stearic acid per 100 gm food (equivalent to 14.94 gm of stearic acid and 76 mg of calcium).

[3] Including standard error of mean calculated as in table 3, footnote 2.

It is surprising that, although the total level of metabolic fat is about the same on diets E and F, the quantity per gram of stool produced on the calcium-magnesium-low diet is more than double that obtained when the diet contains these salts; this is attributed to the marked reduction in the weight of the stools excreted in the former case. The value found for diet E (65 mg) corresponds quite well with that determined by Augur et al. ('47), which was 50.5 mg.

Experiments on the digestibility of rapeseed oil,[5] bland lard,[6] and several samples of hydrogenated lard are summarized in table 3, while table 4 records the results of the tests with synthetic triglycerides.[7] Data on the absorption of palmitic and stearic acids fed as such with or without the calcium-magnesium salts or as the calcium soap are given in table 5.

DISCUSSION

The data presented offer some indication that an inverse relationship exists within a certain range between digestibility and melting point. Thus, in the series of tests with simple triglycerides where calcium and magnesium were present, the coefficients of digestibility were as follows: Trilaurin (m.p., 47.8°C.) 77.2; trimyristin (m.p., 56°C.) 37.7; tripalmitin (m.p., 66.5°C.) 12.8; and tristearin (m.p., 70°C.) 10.6. The values for the samples of blended and hydrogenated lard show somewhat higher digestibility in the lower range of melting points but approach the low level in the sample with m.p. of 61°C.

[5] The rapeseed oil was obtained from the Pacific Vegetable Oil Company and was the same sample employed in earlier tests (Deuel, Cheng and Morehouse, '48). The various lard samples were furnished by Swift and Company and were some of the same samples used in the tests of Crockett and Deuel ('47). Synthetic triglycerides were Eastman products, while the monostearin was a commercial product furnished by Dr. H. W. Vahlteich of The Best Foods Corporation. The monostearin had a saponification number of 156.7 (theory 156.2). The calcium salts of palmitic and stearic acids were prepared from the corresponding fatty acids by slow precipitation with a dilute $CaCl_2$ solution, followed by filtration and thorough washing with acetone and drying under a vacuum at 50°C.

[6] See footnote 5.

[7] See footnote 5.

The results were as follows: Bland lard (m.p., 47.8°C.) 92.4; hydrogenated lard and blended lard (m.p., 55.4 and 55.2°C.), 58.0 and 66.2; and hydrogenated lard (m.p., 61.0°C.) 17.3. The results of the tests where the calcium and magnesium content was at a minimum show similar relationships. These results are evident from an inspection of figure 1, where digestibility is plotted against melting point.

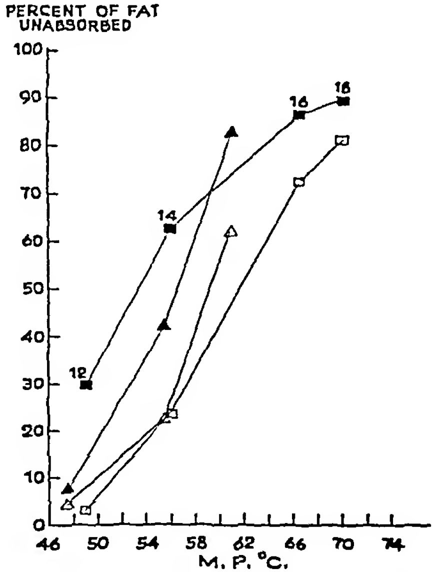

Fig. 1 The relationship of melting point to digestibility of simple triglycerides (■) and of samples of hydrogenated lard (▲), where calcium and magnesium were present in the diet. The results of tests with calcium-magnesium-low diets is represented in each case by similar characters which are not filled in. The numbers by each character indicate the number of carbon atoms in the fatty acid component of the simple triglycerides.

The present results do not confirm the earlier experiments of Lyman ('17) or Hoagland and Snider ('43), who reported that tripalmitin is digested to an amount greater than 80% in dogs and rats. In the present tests it was found that tripalmitin was absorbed only to the extent of 12.8% in rats, while this was increased to 27.9% when calcium and magnesium were removed from the diet. The low digestibility of tripalmitin in

our experiments is further confirmed by the results when the free acid or soap was fed; in these cases the coefficients of digestibility were 19.8 and 16.7, respectively. In the absence of calcium and magnesium this figure was increased only to 35.6. The reason for the discrepancies between our work and that of the earlier investigators is not immediately evident. In our tests no supplementary oil was given in the diet, while in those of Hoagland and Snider ('43) olive oil was present and must have dissolved the palmitic acid to a considerable extent.

The presence of calcium and magnesium in the diet does not change the digestibility of the natural fats with lower melting points. Thus the following high digestibilities have been reported by Crockett and Deuel ('47) for fats melting under 50°C. when fed with the high calcium-magnesium diet: Margarine (m.p., 34°C.) 97.0; commercial hydrogenated cottonseed oil[8] (m.p., 43°C.) 97.3; prime steam lard (m.p., 37°C.) 96.6; and bland lard (m.p., 48°C.) 94.3. On the other hand, the removal of calcium and magnesium from the diet markedly increases the digestibility of the fats with higher melting points although the effect becomes much less pronounced in the case of tristearin. In the calcium-magnesium diet, trilaurin is digested to the extent of 70.5%, while in the absence of these divalent ions the value rises to 97.3. Corresponding coefficients of digestibility for trimyristin under these divergent dietary conditions are 37.7 and 76.6, respectively, while with tristearin the values are 10.6 and 18.9. That this effect is related to a mass action phenomenon is indicated by the trilaurin tests, where the addition of smaller proportions of calcium and magnesium to the diet resulted in a lessened depressive action on digestibility.

When calcium and magnesium were omitted from the diet, a marked decrease in the proportion of fat excreted as soap occurred, while some increase in the neutral fat-fatty acid fraction usually resulted. This effect was most marked with trilaurin and trimyristin and in the cases of the natural and

[8] Crisco.

hydrogenated fats studied. The shift from the soap to the neutral fat-fatty acid fraction on the elimination of calcium and magnesium from the diet was much less pronounced in the case of the triglycerides; the bulk of the fat excreted even when calcium and magnesium were present in the diet was in the neutral fat-fatty acid fraction. That the low absorption of tripalmitin and tristearin is not solely the result of a failure in lipolysis is shown by the fact that the fatty acid content of the neutral fat-fatty acid fraction was 35% in the tripalmitin tests and 32% in the tristearin experiments. As the insolubility of the fatty acids increases, the tendency to form soaps is diminished. This may be the result of the extreme insolubility of the free acid plus a very low degree of ionization.

These data confirm the earlier report of Boyd, Crum and Lyman ('32) on the differential effect of the level of calcium intake in depressing the fat absorption as related to the fatty acid make-up of the diet. Conversely, the calcium intake level is of considerable importance in relationship to calcium retention as a function of the fat content of the diet. It is evident that a minimum calcium loss will occur when the dietary fats have low melting points; however, when the melting point of the ingested fat exceeds 50°C., one may expect a marked loss of calcium, proportional to the amount of such fat fed.

The elimination of calcium and magnesium from the diet markedly lowers the weight of the stools in all cases except those of tripalmitin and tristearin. In many cases stools from rats on calcium-magnesium-low diets weigh less than 50% of those where these ions are included in the rations.

SUMMARY

1. In a series of tests with simple triglycerides an inverse relationship obtained between the melting point and the coefficient of digestibility. A similar relationship was noted with hydrogenated lards of varying melting points.

2. Although the presence of calcium and magnesium in the diet does not influence the digestibility of low-melting fats, it markedly decreases that of higher-melting simple triglycerides and hydrogenated fats. The digestibilities with and without the divalent ions were as follows: Trilaurin, 70.5 and 97.3; trimyristin, 37.7 and 76.6; tripalmitin, 12.8 and 27.9; and tristearin, 10.6 and 18.9. With respect to several lards the corresponding coefficients of digestibility with and without the calcium-magnesium salts were as follows: Bland lard (m.p., 47.8°C.), 92.4 and 95.8; hydrogenated lard (m.p., 55.4°C.) 58.0 and 77.9; blended hydrogenated lard (m.p., 55.2°C.) 66.2 and 80.0; and hydrogenated lard (m.p., 61°C.) 17.3 and 38.0.

3. The absence of calcium and magnesium from the diet markedly increases the neutral fat-fatty acid fraction at the expense of the soap fraction. This effect is most marked with trilaurin, trimyristin, and the hydrogenated lard samples, and least with tripalmitin and tristearin. In the latter case the bulk of the excreted fat under either dietary regime is in the neutral fat-fatty acid fraction.

4. The effect of calcium and magnesium was a progressive one, being greater the larger the proportion of these salts in the diet.

5. In all cases except in the tests with tripalmitin and tristearin the elimination of calcium and magnesium from the diet resulted in a decrease in stool weight of 50% or more.

6. The removal of calcium and magnesium from the diet resulted in an increase in the digestibility of crude and refined rapeseed oil to 92 and 93%, respectively.

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EFFECT OF OATS, OAT PRODUCTS AND FAT ON THE INTESTINAL SYNTHESIS OF BIOTIN IN MATURE FOWL [1]

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TWELVE FIGURES

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The laying hen has been used as an experimental animal by Couch and associates ('48a) for studying the intestinal synthesis of biotin as reflected (1) by the biotin content of the egg and (2) by hatchability. These workers reported that dextrin favored the intestinal synthesis of this vitamin but that sucrose did not promote such synthesis, and further that lactose and dried whey did not stimulate the synthesis of biotin in the intestinal tract of the laying fowl. Congenital anomalies in the chick resulting from feeding hens a low biotin diet have been described by Cravens et al. ('44) and by Couch et al. ('48b).

The present investigation was initiated to determine the effect of oats, oat products and fat on the intestinal synthesis of biotin in the mature fowl. Hatchability of fertile eggs, incidence of congenital anomalies, and biotin content of the eggs were criteria used in evaluating the extent of intestinal synthesis and absorption of biotin.

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MATERIALS AND METHODS

The management and general experimental procedure was the same as that previously reported by Couch et al. ('48a). The composition of the diets is shown in table 1. Crystalline B-vitamins with the exception of biotin and pteroylglutamic

TABLE 1

Composition of diets

	B31	B31A	B32	B32A	B34	B45	B45A	B46	B46A	B47	B48
	%	%	%	%	%	%	%	%	%	%	%
Sucrose	63	63			63	43	23	43	23	53	53
Dextrin		.	63	48				..	..	..	..
Ground whole oats		..				20	40		..	..	.
Ground oat groats		.						20	40	..	..
Ground oat hulls									.	10	10
Purified casein	18	18	18	18	18	18	18	18	18	18	18
Gelatin	5	5	5	5	5	5	5	5	5	5	5
Salts IV	5	5	5	5	5	5	5	5	5	5	5
Liver fraction "L"	4	4	4	4	4	4	4	4	4	4	4
Fish oil (3000A) (400D)	2	2	2	2	2	2	2	2	2	2	2
Soybean oil	3	3	3	18	3	3	3	3	3	3	7
Oyster shells	Ad libitum										
Vitamins and choline listed below added in mg per kg of diet											
Biotin	..	0.016	.		0.2		..	..	..	..	..
Thiamin HCl	4	4	4	4	4	4	4	4	4	4	4
Riboflavin	6	6	6	6	6	6	6	6	6	6	6
Ca pantothenate	15	15	15	15	15	15	15	15	15	15	15
Niacin	100	100	100	100	100	100	100	100	100	100	100
2-methyl-1,4-napthoquinone	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine HCl	4	4	4	4	4	4	4	4	4	4	4
Alpha-tocopherol	3	3	3	3	3	3	3	3	3	3	3
Choline	200	200	200	200	200	200	200	200	200	200	200

acid were added in amounts which were thought to meet the requirements of the birds. Liver fraction "L" was used as a source of the latter vitamin and also as a source of unidentified factors. Biotin was added to diets B31A and B34. Oyster shells and tap water were supplied ad libitum. The oat

products used were ground in a burr mill to a fineness which made for uniform distribution in the basal diet.

The pullets were fed an all-mash laying ration for 15 weeks prior to the start of the first experiment, during which period data were collected on egg production and hatchability. At the end of this pre-experimental period 16 birds were selected and divided into 4 groups of 4 birds each. The effect of 20% whole oats (B45), 20% oat groats (B46) and 10% oat hulls (B47) on the intestinal synthesis of biotin was studied by using diet B31 as a negative control and replacing an equivalent amount of sucrose with each of the above-mentioned ingredients. Two other groups of 4 hens, each of which had been used in an earlier experiment, were continued on diet B34 and the practical all-mash diet. The latter two groups served as positive controls in this experiment. The duration of the experimental period was 5 weeks.

Eight groups of 4 hens each were used in experiments 2 and 3; these were conducted concurrently. In experiment 2 the percentage of whole oats (diet B45A) and oat groats (diet B46A) was increased to 40; one group (diet B31A) was included in which 0.016 mg of crystalline biotin per kilogram of diet was added (equivalent to biotin in 40 parts of whole oats). Diet B47 (10% oat hulls) was repeated; and diet B31 was used as the negative control.

In experiment 3 the effect of fat on the intestinal synthesis of biotin was studied by using dextrin as the carbohydrate (B32) and increasing the fat from 5 to 20% (diet B32A). Further information was also obtained on the effect of fat through a comparison of diets B47 and B48. In diet B48 the soybean oil was increased to 7 parts; this makes diet B48 equal to diet B31 from a caloric standpoint without taking the oat hulls into account. Since experiments 2 and 3 were conducted concurrently and under the same conditions, data from hens fed diets B31 and B47 were included in both experiments. Hens fed diet B31 served as negative controls in both instances. In addition, hens fed diet B34 and the practical

all-mash diet (described above) were again used as positive controls. The duration of these experiments was also 5 weeks.

Data were also included from a 4th experiment in which one group of hens was fed diet B31 and another was fed diet B40, which was the same as B31 except that 5 parts of cellu-flour were substituted for an equivalent amount of sucrose.

Eggs were set aside for biotin determinations on the last two days of each week. A modification of the method of Wright and Skeggs ('44), in which turbidity was the criterion employed in measuring the growth of *Lactobacillus arabinosus,* was used in determining the biotin content of the eggs. The biotin content of the egg yolks and whites was expressed in millimicrograms per gram on a fresh weight basis. The biotin content of the oats and oat products was also determined.

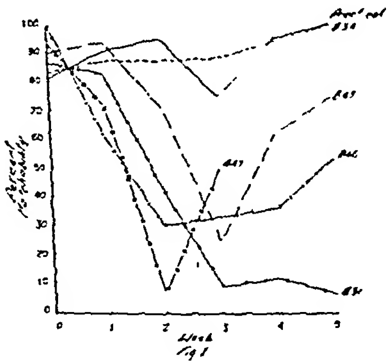
During the course of the studies herein reported, biotin determinations were also made on feces from hens fed the practical all-mash diet.

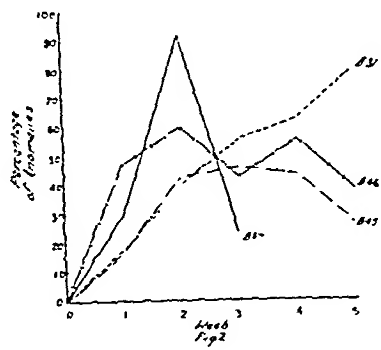
It was found that symptoms of biotin deficiency could be ascertained in a 12-day-old embryo. All such embryos which died were used in determining the percentages of each on various diets that were affected with congenital anomalies traceable to a deficiency of biotin in the maternal diet.

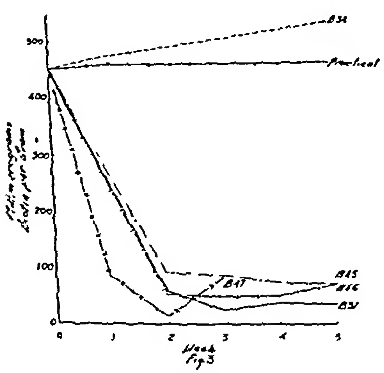
RESULTS AND DISCUSSION

This investigation has shown that the feeding of ground whole oats promotes the intestinal synthesis of biotin when substituted for sucrose in a low biotin diet. When 20% whole oats was used (diet B45), the per cent of hatchability throughout the test was higher than that obtained with diet B31 (fig. 1). In the first experiment the hens fed diet B45 appeared to undergo an adjustment (possibly in the intestinal flora) during the first three weeks, at the end of which time the per cent of hatchability was 25. However, after such an adjustment, the hatchability of eggs from hens fed diet B45 increased to 75% at the end of the fifth week. The feeding of 20% whole oats likewise increased the biotin content of

the yolks and whites of eggs over that observed for eggs from hens fed diet B31 (figs. 3 and 4) and produced a smaller percentage of anomalies than in embryos and chicks from hens fed diet B31. The use of 20% oat groats (B46) in a low biotin diet also appeared to exert a favorable effect on the intestinal synthesis of biotin when hatchability (fig. 1), congenital deformities (fig. 2) and the biotin content of the eggs (figs. 3







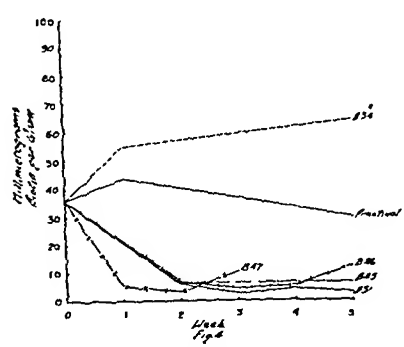


Fig. 1 Effect in hatchability of adding oats and oat products to a low biotin diet: diet B31, sucrose; diet B34, biotin; diet B45, 20% oats, diet B46, 20% oat groats; diet B47, 10% oat hulls.

Fig. 2 The incidence of congenital deformities as influenced by the addition of oats and oat products to a low biotin diet for breeding hens.

Fig. 3 Effect on the biotin content of egg yolk of adding oats and oat products to a low biotin diet.

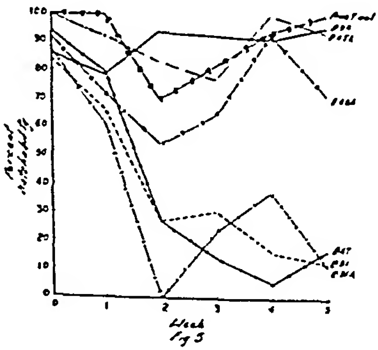
Fig. 4 Effect on the biotin content of egg white of adding oats and oat products to a low biotin diet.

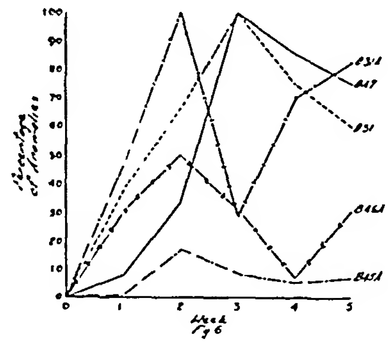
and 4) were considered, and a comparison was made with the results obtained with diet B31. The favorable effect on the intestinal synthesis of biotin appeared to be slightly greater in the case of the hens fed whole oats than in that of those fed oat groats. The results obtained through the feeding of oat hulls (B47) in the first experiment are somewhat erratic and inconclusive, due in part to the fact that the hens fed this diet stopped laying at the end of the third week of the test (figs. 1, 2, 3 and 4).

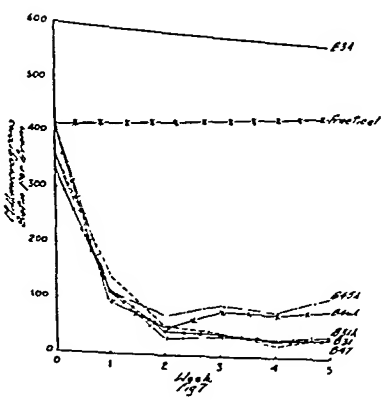
Data on egg production were not included in the study reported herein due to he fact that the percentage egg production was approximately the same in all cases, with the exception of the hens fed 10% oat hulls (diet B47) in the first experiment. In this case the hens stopped laying about the end of the third week of the experimental period. However, hens fed this diet (B47) in the second experiment laid at the same rate as those of other groups. Thus the observation on egg production of hens fed diet B47 in experiment 1 was not considered to be of any significance.

In the second experiment more conclusive results were obtained by increasing the percentage of whole oats and oat groats to 40. Apparently normal hatchability (fig. 5) was obtained by using 40% whole oats (B45A) in a low biotin diet. Some of the embryos and day-old chicks exhibited symptoms of biotin deficiency (fig. 6), indicating that the amounts of biotin which were absorbed and deposited in the egg were not optimum. This is also indicated by the biotin values of egg yolks and whites (figs. 7 and 8). Further evidence for the fact that whole oats supported the intestinal synthesis, absorption and deposition of biotin in the egg may be obtained by comparing the results on hatchability (fig. 5), congenital deformities (fig. 6), and the biotin content of the eggs (figs. 7 and 8) obtained by feeding 40% whole oats (B45), with those obtained by feeding diet B31A to which was added 0.016 mg of crystalline biotin (equivalent to that amount of the vitamin contributed by 40 parts of whole oats).

Forty per cent oat groats (diet B46A) favored the intestinal synthesis of biotin to a lesser extent than 40% whole oats (diet B45A, figs. 5, 6, 7, and 8). The fact that diet B46A supported the synthesis of the vitamin may be seen by comparing the per cent of hatchability (fig. 5), percentage of anomalies







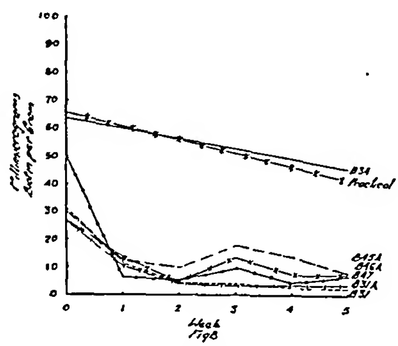


Fig. 5 Effect on hatchability of adding oats and oat products to a low biotin diet: diet B31, sucrose; diet B31A, 0.016 mg biotin per kilogram; diet B34, 0.2 mg biotin per kilogram; diet B45A, 40% oats; diet B46A, 40% oat groats; diet B47, 10% oat hulls.

Fig. 6 The incidence of congenital deformities as influenced by the addition of oats and oat products to a low biotin diet for breeding hens.

Fig. 7 Effect on the biotin content of egg yolk of adding oats and oat products to a low biotin diet.

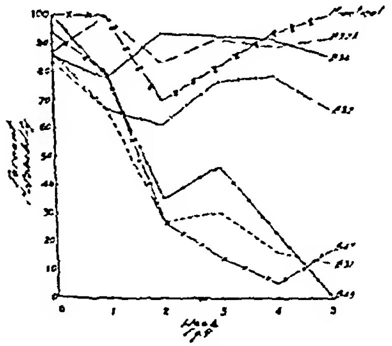
Fig. 8 Effect on the biotin content of egg white of adding oats and oat products to a low protein diet.

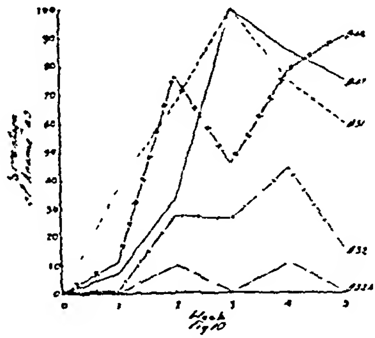
(fig. 6), and biotin content of the egg yolks and whites (figs. 7 and 8) which resulted from feeding hens diet B46A with the data obtained when hens were fed diet B31.

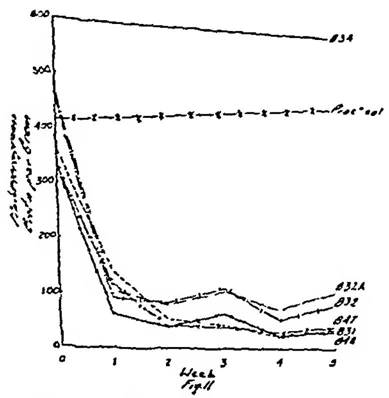
The use of 10% oat hulls (diet B47) in a low biotin diet did not favor intestinal synthesis of biotin as far as hatchability (fig. 5), incidence of congenital deformities (fig. 6) or the biotin content of the egg yolks and whites (figs. 7 and 8) are concerned.

From the results of the third experiment (figs. 9, 10, 11, and 12) it may be noted that dextrin with 5% fat (diet B32) or 20% fat (diet B32A) supported the intestinal synthesis of biotin. This may be seen by comparing the results obtained by feeding diets B32 and B32A with those obtained by feeding diet B31 with respect to hatchability (fig. 9), percentage of anomalies (fig. 10) and biotin content of egg yolks and whites (figs. 11 and 12). This is in agreement with the work of Couch et al. ('48a), in which it was reported that dextrin favored the synthesis of biotin in the intestinal tract of the laying fowl. When the results are judged from the standpoint of hatchability (fig. 9) and percentage of congenital deformities (fig. 10), there is an indication that 20% fat with dextrin (diet B32A) favored the intestinal synthesis of the vitamin to a greater extent than did 5% fat with dextrin (diet B32). However, the difference between the biotin content of egg yolks and whites from hens fed diet B32A and those from hens fed diet B32 were not so striking (figs. 11 and 12). The use of 10% oat hulls in a diet with either 5 (B47) or 9 parts of fat (B48) did not appear to have any appreciable effect on the intestinal synthesis of biotin (figs. 9, 10, 11 and 12). It is possible that the 10 parts of oat hulls used in the diet could not overcome the effect of the principal carbohydrate (sucrose) used in diets B47 and B48, which does not support the synthesis of the vitamin in the intestinal tract of the fowl. Davis and Briggs ('47) reported that the addition of 5, 10 and 15% cellulose to a purified chick diet resulted in a significant increase in growth. However, these workers used "cerelose" as the source of carbohydrate. Five per cent cellu-flour failed

to have any effect on the intestinal synthesis of biotin in the 4th experiment of the present study. However, again it should be pointed out that sucrose was the carbohydrate, and it does not favor the synthesis of biotin in the alimentary tract of the laying fowl. It was observed in the present investigation that whole oats favored the intestinal synthesis of biotin in







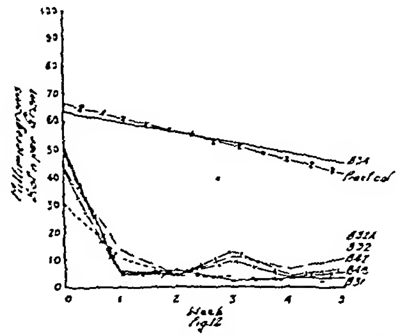


Fig. 9 Effect on hatchability of fat additions to a low biotin diet: diet B31, sucrose; diet B32, dextrin; diet B32A, dextrin with 20% fat; diet B34, 0.2 mg biotin per kilogram; diet B47, 10% oat hulls; diet B48, 10% oat hulls with 9 parts fat.

Fig. 10 Effect of fat additions to a low biotin diet on the incidence of congenital deformities.

Fig. 11 Effect of fat additions to a low biotin diet on the biotin content of egg yolk.

Fig. 12 Effect of fat additions to a low biotin diet on the biotin content of egg white.

the mature fowl to a greater extent than did oat groats. It is possible that the hulls fed with the oats provided a more favorable medium for the growth of intestinal microorganisms than that provided by oat groats. If this is the case, the addition of a fibrous product to a dextrin diet such as B32 might further enchance the synthesis of biotin.

It would be of considerable interest to determine the effects of feedstuffs other than oats and oat products on the intestinal synthesis of biotin. Some data bearing on this subject were obtained by running essentially a balance trial with hens fed the practical all-mash diet. From these results it was determined that a hen ingested about 10 μg of biotin per day and excreted approximately 20 μg of the vitamin in the feces. If an egg was laid an additional 6 μg of biotin could be accounted for. Thus it is apparent that in the case of laying hens on a practical all-mash diet, approximately two and one-half times the quantity of the vitamin in the food ingested can be accounted for through excretion in the feces and deposition in the egg. The portion of the gastrointestinal tract where this synthesis of biotin occurs is a problem under investigation at present.

SUMMARY

Evidence is presented to show that ground whole oats and oat groats supported the intestinal synthesis of biotin in the mature fowl when used to replace either 20 or 40% of sucrose in a low biotin diet. The synthesis, absorption and deposition of the vitamin in the egg were directly related to the level of whole oats or oat groats used. The results obtained indicate that whole oats stimulated the synthesis of the vitamin to a greater extent than did the oat groats. Oat hulls did not favor the intestinal synthesis of biotin under the conditions of this experiment.

An indication was obtained to the effect that 20% fat with dextrin favored the intestinal synthesis of biotin to a greater extent than 5% fat with dextrin.

ACKNOWLEDGMENTS

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THE IMPORTANCE OF BULK IN THE NUTRITION OF THE GUINEA PIG[1]

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In spite of the rather extensive studies on the nutritional requirements of the guinea pig (cavy) it still is not possible to devise a purified ration that will produce "normal" rates of growth. Cannon et al. ('45) reported that growth of less than 7 to 8 gm per day from the second to the 8th weeks of life cannot be considered "normal" if growth on natural foodstuffs is accepted as the standard. These same workers concluded that growth and survival on purified rations, supplemented with linseed oil meal and solubilized liver, was poorer than on a crude diet and that the animals fed the former developed anemia and leucopenia. Woolley and Sprince ('45) described three guinea pig factors (abbreviated, G.P.F.) numbered 1, 2, and 3. They stated that G.P.F.-1 was folic acid, G.P.F.-2 was replaceable by a mixture of cellulose and protein, and G.P.F.-3 was an as yet unidentified factor present in solubilized liver extracts. However, the best growth obtained when all three of these factors were added to a purified basal ration was only 4 gm per day during a period of 4 weeks.

[1] Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. This work was supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation; also by funds granted by the National Dairy Council, Chicago, on behalf of the American Dairy Association.

The guinea pig, unlike the rat, is an herbivorous animal, subsisting entirely on foods of plant origin. Therefore, it is not surprising to find that the digestive tract is different, in that it includes a large functioning caecum constituting about 15% of the body weight. The results of the present investigation will be cited in an attempt to relate this peculiarity in the lower intestinal tract to the importance of bulk in a purified ration.

EXPERIMENTAL

There is abundant evidence to indicate that the guinea pig is very susceptible to certain diseases, especially *Salmonella* infections and pneumonia. For this reason two precautions were taken: first, the animals were obtained from a single source during the entire investigation and, secondly, the animals were housed in a room of uniform temperature (75 to 85°F.), in individual wire bottom cages which were steam sterilized before being used. Water was supplied by means of bottles hung on the outside of each cage with a glass tube and drinking tip on the inside of the cage.

A commercial ration [2] was used as the positive control diet. It is known to be high in alfalfa leaf meal and has always resulted in excellent growth when used for our animals. The basal synthetic diet had the following composition: Sucrose, 60; casein [3] (vitamin free), 30; salts IV, 4; fortified soybean oil, 4; sucrose mixture containing B vitamins, 0.8; and choline, 0.3 parts. The fortified soybean oil was prepared by dissolving β-carotene in chloroform and distributing it as a layer on the sides of a round-bottom flask while removing all of the chloroform with reduced pressure. Soybean oil was added to the flask and heated (60–70°) on a sand bath for three or 4 hours until the carotene was dissolved in the oil. The solution was allowed to cool and crystalline vitamin D_2 and vitamin K as menadione were added. Finally alpha-tocopherol was added and the fortified oil made up to a final weight. The use

[2] Rockland guinea pig pellets.

[3] Obtained from General Biochemicals, Inc., Chagrin Falls, Ohio.

of 4% of this oil supplied 1.2 mg of β-carotene, 12 mg of α-tocopherol, 8 μg of calciferol, and 0.2 mg of vitamin K per 100 gm of ration. The added mixture of B vitamins supplied 1 mg of thiamine hydrochloride, 1.4 mg of riboflavin, 1 mg pyridoxine, 3 mg calcium pantothenate, 200 mg inositol, 10 mg niacin, 10 mg *p*-aminobenzoic acid, 0.04 mg biotin, and 0.3 mg folic acid per 100 gm of ration. Vitamin C was supplied by dissolving sufficient ascorbic acid in a 70% sugar solution to secure 100 mg per milliliter. This solution was fed with individual pipettes at a level of 0.25 ml every other day, which was equivalent to 12.5 mg per animal per day.

Loss of guinea pigs during the first few weeks on experiment was completely eliminated in most groups of animals if small amounts of the stock ration were placed on top of the experimental diet during the first 7 days. Approximately 4 or 5 gm were used the first day and the amount decreased each day until no addition was made after the 7th day. In spite of these precautions, a high mortality was observed in a few of the experimental groups. No explanation could be found for these results, and when the condition was encountered the entire group of animals was discarded. It should be emphasized that animals from these groups never failed when fed the stock ration.

RESULTS

The growth data are given in table 1. Since the rate of growth of the animals in different groups varied to some extent, the results are separated into several series. When the commercial stock ration, supplemented with vitamin C, was fed, an average daily gain in weight of 6.9 gm was obtained over a 6-week period. The basal synthetic ration, on the other hand, which contained all of the known dietary factors except vitamin B_{12}, gave a growth response of only 1.8 gm per day. When crude casein was used in place of the vitamin-free casein the same results were obtained. No improvement resulted when dextrin was used in place of sucrose. Since one of the major components of the stock ration was alfalfa leaf

TABLE 1

Growth of guinea pigs on different rations[1]

	NO. OF ANIMALS	WEEKS ON EXP.	AVERAGE GAIN IN WT.
			gm/day
Series I			
Rockland stock diet	30	6	6.9
Basal synthetic	9	6	1.8
Basal synthetic (crude casein)	7	6	1.9
Basal synthetic (dextrin)	3	6	1.9
Basal synthetic + 15% alfalfa leaf meal	4	6	3.6
Basal synthetic + 25% alfalfa leaf meal	4	8	6.5
Basal synthetic + 25% autoclaved alfalfa leaf meal	5	6	6.5
Basal synthetic + 30% beet pulp	4	6	6.8
Basal synthetic + 10% beet pulp fiber	4	6	2.0
Basal synthetic + 20% beet pulp fiber	2	6	3.1
Basal synthetic + 20% pectin	3	6	4.4
Basal synthetic + 20% gum arabic	3	6	5.2
Basal synthetic + 15% cellu flour	9	6	4.6
Series II			
Basal synthetic + 15% gum arabic	20	6	5.1
Basal synthetic + 15% xylose	3	3	3.5
Basal synthetic + 2% levulenic acid	4	4	2.5
Basal synthetic + hydrolyzed gum arabic ≅ 15%	5	4	2.5
Series III			
Basal synthetic + 15% gum arabic + summer milk ad lib.	3	6	4.7
Basal synthetic + 15% gum arabic + extract of beef liver ≅ 10%	5	6	5.7
Basal synthetic + 15% gum arabic + 15% brewers' yeast	5	6	6.7
Basal synthetic + 15% gum arabic + 4% fish solubles	6	6	2.5
Basal synthetic + 15% gum arabic + 6% grass juice powder	6	6	4.6
Basal synthetic + 15% gum arabic + 4% grass juice	6	6	2.8

TABLE 1 *(continued)*

	NO. OF ANIMALS	WEEKS ON EXP.	AVERAGE GAIN IN WT.
			gm/day
Series IV			
Rockland stock diet	4	9	6.6
Basal synthetic + 15% gum arabic	5	9	5.1
Basal synthetic + 15% gum arabic + alfalfa ash ≅ 25%	5	9	5.4
Basal synthetic (crude casein) + 15% gum arabic	5	9	5.5
Basal synthetic (crude casein) + 15% gum arabic + alfalfa ash ≅ 25%	6	9	7.0
Basal synthetic (crude casein) + 15% gum arabic + stock ration ash ≅ 100%	4	9	5.7
Basal synthetic + alfalfa ash ≅ 25%	5	9	4.4
Series V			
Basal synthetic + 15% gum arabic	10	6	5.1
Basal synthetic (20% casein) + 15% gum arabic	10	6	2.3
Basal synthetic (20% crude casein) + 15% gum arabic	9	3	2.4
Basal synthetic (20% casein) + 15% gum arabic + 0.3% L-cystine + 0.1 DL-tryptophan	5	6	4.1
Basal synthetic (20% casein) + 15% gum arabic + 1% DL-arginine + 0.1% L-cystine + 0.2% glycine	2	6	2.5
Basal synthetic (15% casein) + 15% gum arabic + 15% wheat gluten	3	6	4.7
Basal synthetic (8% casein) + 15% gum arabic + 8% fibrin + 8% egg albumin	3	6	4.2
Basal synthetic (20% casein) + 15% gum arabic + 7% protolysate	4	3	2.7
Basal synthetic (20% casein) + 15% gum arabic + extract of crude casein ≅ 50%	6	3	3.0
Basal synthetic (30% special casein) + 15% gum arabic	5	4	5.1
Basal synthetic (18% casein) + 30% beet pulp	4	6	5.1
Basal synthetic (28% casein) + 30% beet pulp	4	6	5.2

[1] All additions made at the expense of the sucrose in the basal ration.

meal, experiments were set up to determine if the addition of this material would improve the growth of the animals. When 25% of leaf meal was added to the basal synthetic ration the average rate of growth was equal to that obtained on the stock ration, but 15% of the meal was definitely inadequate. Apparently alfalfa supplied all of the missing dietary factors when added at the higher level. The effect of the alfalfa was not altered when the meal was autoclaved.

Woolley and Sprince ('45) recognized dietary bulk as an important factor in the growth of guinea pigs and used cellu flour and cellophane to satisfy this requirement. In order to study the effect of bulk more extensively, it was decided to try beet pulp, a material which contains more than 20% crude fiber. Plain dried beet pulp was added to the ration at a level of 30%. When this addition was made the gain in weight over a 6-week period was equal to or better than that obtained with 25% alfalfa leaf meal. However, when the crude fiber from beet pulp was isolated and fed at levels of 10 and 20%, poor growth resulted. These results were somewhat unexpected and led to an examination of the chemical analysis of beet pulp. It was found that beet pulp contains as much as 30% of pectin, which is a hemicellulose (Codling and Woodman, '29).

The following materials were therefore tested in order to differentiate between the effects of cellulose and hemicellulose: Cellu flour, cellophane, pectin,[4] gum arabic, gum mesquite, gum tragacanth, oat straw, agar, cornstarch, potato starch, paper pulp, wood shavings, silica gel and methyl cellulose. Of all of these materials, powdered gum arabic consistently produced the best response. Pectin, agar, oat straw, cellu flour and cellophane stimulated growth to some extent but to a lesser degree than gum arabic. The other materials were either inactive or only slightly active.

Many of the animals receiving the basal ration plus gum arabic were continued on the ration after the 6-week period,

[4] Obtained through the courtesy of the California Fruit Growers Exchange, Ontario, California.

and these animals continued to grow and showed no indication of a serious deficiency during a period of 8 months or more. Post-mortem examination of guinea pigs failing to survive on the basal ration without gum arabic revealed no specific abnormalities. One symptom which was noted was that the animals ate the hair on the abdomen and sides of the body. This was observed in animals on almost all the inadequate rations but never in more than two out of 5 cavies on the same ration. Another symptom which was frequently observed on adequate as well as inadequate diets was diarrhea. The duration and severity of the condition varied greatly. Occasionally rapidly growing animals would suddenly lose as much as 50 gm in body weight, at which time a severe diarrhea developed. However, all of the animals on any given ration did not exhibit such tendencies. The feces from animals receiving the stock rations were always hard and dry in appearance, whereas the feces from guinea pigs on purified rations were unusually soft, even when the rate of growth was normal.

When it was found that gum arabic was active in promoting growth there were at least two questions to be answered; first, how does the gum function and, secondly, are there any other missing dietary factors to be contended with when a ration containing 15% gum arabic is fed? Gum arabic contains 28.3% galactosoglucuronic acid, 29.5% galactose, 34.4% arabinose and 14.2% rhamnose hydrate. Arabinose was not available, but in order to test the activity of a pentose sugar, xylose [5] was fed at a level of 15%. Poor growth resulted (series II). Davis and Briggs ('47) reported that materials like Ruffex, wood shavings, and cellu flour were capable of stimulating the growth rate of chicks when added to a purified ration at levels of 5 to 15%. These investigators also found that the degradation products of cellulose, furfural and levulenic acid, were equally active. Guinea pigs were fed a ration containing 2% levulenic acid but negative results were obtained. The most critical experiment designed to determine

[5] Obtained through the courtesy of the Northern Regional Research Laboratory, Peoria, Illinois.

whether the gum could be replaced by degradation products involved hydrolysis of gum arabic with dilute H_2SO_4 (Butler and Cretcher, '29), removal of the sulfate with $CaCO_3$, and then addition of the filtrate to the purified ration. Again the results, as judged by the average gain in weight, were subnormal and it was concluded that the intact gum molecule was essential for growth-promoting activity, probably due to a "bulk" effect on the lower intestinal tract.

The average growth rate for 20 animals receiving a ration containing 15% gum arabic was 5.1 gm per day (series II). Since a growth rate of 6.9 gm per day was obtained on the stock ration, it appeared that gum arabic was not the only factor involved. Various natural supplements were added to a ration containing 15% gum arabic, including raw summer milk (ad libitum) fish solubles, grass juice powder,[6] grass juice (20% solids),[7] a liver extract and yeast (series III). Yeast produced a good response and the methanol extract of beef liver produced some response but the other supplements, namely milk, fish solubles, grass juice powder, and grass juice, appeared to depress rather than enchance growth.

Since 25% alfalfa leaf meal was capable of bringing about a normal growth rate, fractionation of this material was undertaken. It was found that an ether extract, a 90% ethanol extract, a methanol extract, or hot water extracts were all less active than the residues from which these extracts were prepared. The next logical fraction to test was the ash. Surprisingly enough, the ash of alfalfa leaf meal showed some effect when added to purified rations either with or without gum arabic (series IV). In fact, nearly normal growth rates were obtained when a ration containing 15% gum arabic plus alfalfa ash equivalent to 25% was fed. This ration has been tested several times and the results were nearly always in the normal range during the third to the 6th week of the experiment. The growth rate was often subnormal for the first three

[6] Obtained through the courtesy of the Cerophyl Laboratories, Kansas City, Missouri.

[7] See footnote 5.

weeks of an experiment, after which nearly normal growth occurred. Whether this initial lag in growth is due to a missing dietary essential or simply a refusal on the part of the guinea pig to accept this ration readily is not known.

Stock ration ash as the only source of minerals in the ration (the ash from 100 gm of stock ration was substituted for 4 gm of salts IV) was as effective as alfalfa ash. The use of crude casein had some beneficial effect, especially in the presence of alfalfa ash. However, when the animals were continued on this ration for 12 weeks there was little difference between the animals receiving the purified and the crude casein.

Attempts have been made to determine what particular constituent in the alfalfa accounts for the activity observed. Cobalt, boron and molybdenum have been added singly and together without success. Sodium, magnesium, calcium and phosphorus have also been eliminated as being involved. The level of salt mixture in the basal ration (salts IV, Hegsted et al., '41) was raised from 4 to 8% with no improvement. The ash of alfalfa is quite basic and this may exert a favorable effect on some of the microflora of the lower tract by raising the pH. Another possibility which must be explored is the sodium-potassium ratio, because alfalfa ash contains a high level of potassium. Further work is in progress to clarify this question.

Van Wagtendonk and Wulzen ('43) observed the development of a wrist-stiffness in guinea pigs on a ration containing vitamin-fortified skim milk powder plus wheat straw. The deficiency could be corrected by feeding fresh cream or cane juice or crystalline material isolated from these sources. This wrist-stiffness has never been observed in the investigations reported here, and there was no response as measured by growth when either ergostanol[8] (Oleson et al., '47) or the Wulzen anti-wrist stiffness factor[9] was added to a purified ration. The

[8] Obtained through the courtesy of the Lederle Laboratories, Pearl River, New York.

[9] Obtained through the courtesy of the Lilly Research Laboratories, Indianapolis, Indiana.

possibility also arose that vitamin P active compounds such as hesperidin might be stimulatory for growth, but here again negative results were obtained when this substance was tried.

One other question was investigated, namely, the necessity for a high level of casein (30%) in the basal ration. If the level of casein was reduced from 30 to 20% in a ration containing 15% gum arabic, the growth was markedly reduced (series V). It will be recalled that Woolley and Sprince ('45) recognized this fact and they were able to secure growth equal to that obtained with 30% casein by adding arginine (1.0%), cystine (0.1%), and glycine (0.2%) to a ration containing 20% casein. The addition of several amino acids to the 20% casein ration did not improve growth (series V). The commercial stock ration contains only 18% crude protein and excellent growth is always obtained when it is fed. Why then should purified rations containing 20% casein fail? Kuiken et al. ('44) concluded that crude casein contained an unknown dietary factor essential for guinea pigs. Several other experiments were conducted including the addition to purified rations containing gum arabic of wheat gluten, a pancreatic digest of casein,[10] fibrin, egg albumin, and laboratory-prepared cold acetic acid precipitated casein. None of these supplements was able to increase the growth rate to that obtained with 30% casein. Quite recently two rations were compared, both containing 30% beet pulp but one with 18 and the other with 28% crude casein. It was rather surprising to find that the growth rates at the end of 6 weeks were practically the same (5.1 and 5.2 gm per day). These rations also contained dextrin in place of sucrose and cellu flour (3%). However, the fact that the ration containing 18% crude casein produced as good response as the one containing 28% suggests two possibilities: Either the protein (2%) supplied by the 30% beet pulp was an efficient supplement to the casein, or the lower level of protein is adequate when a proper source of bulk is present.

[10] "Protolysate."

DISCUSSION

The results presented in this paper clearly demonstrate the beneficial effect of gum arabic when added to a purified diet for guinea pigs. The exact mechanism by which this gum functions is still unknown. It is known that the class of gums to which this one belongs are hydrophylic in nature and hence have considerable power to retain water and to give colloidal suspensions. Such a gum may function by affecting the type of bacteria multiplying in the lower part of the digestive tract. The gum may favor certain organisms which are able to synthesize unknown factors which are then made available to the host either by direct absorption, by coprophagy or by both mechanisms.

On the other hand, the hemicellulose may tend to decrease the number of unfavorable organisms. Diarrhea which might well owe its origin to an abnormal intestinal microflora, was frequently noted when guinea pigs were fed the unsupplemented rations. Furthermore, the addition of ash from alfalfa has a definite effect and this effect is more apparent in the presence of gum. Further studies are needed to determine which of the mineral elements may be concerned. Perhaps the most important factor is the proper balance among the ash elements that are present in predominant amounts. It is also interesting that the presence of hemicellulose material, especially in the form of beet pulp, tends to decrease the protein requirement. There is little reason to believe that the casein itself supplies a limiting factor; instead, the necessity for the higher level is probably related to the lack of bulk in the diet.

SUMMARY

A purified basal ration containing all of the known nutrients (except vitamin B_{12}) produced a rate of growth of only 1.8 gm per day for 6 weeks compared to a normal rate of 6.9 gm per day when a commercial ration composed of natural feeds was fed. When the purified basal ration was supplemented with 25% alfalfa leaf meal (in place of an equal amount of

sucrose) a nearly normal average growth rate of 6.5 gm per day was obtained. When the basal ration was supplemented with 30% dried beet pulp growth was equally good. Powdered gum arabic (a hemicellulose) was consistently the most active single supplement when fed at a level of 15%. However, the average growth rate of 5.1 gm per day when this gum was fed was less than the normal rate of 6.9 gm. When the ash from alfalfa leaf meal equivalent to 25% was added to a ration containing 15% gum arabic, nearly normal growth rates were obtained for periods as long as 12 weeks. The level of protein in the synthetic ration could be reduced when gum arabic was fed.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

IV. INFLUENCE OF ACTIVITY[1]

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In recently published work from this laboratory Forbes et al. ('46a, '46c), working with growing albino rats, have shown that dietary fat confers economy of food utilization. In their experiments, diets varying in fat content from 2 to 30% were fed so as to supply equal quantities of energy and of protein and a considerable surplus of all known vitamins. Statistically significant results were obtained for gains in nitrogen and energy and decrease in heat production, in the order of increasing fat content of the diets.

In experiments with mature rats, using the same diets fed in isocaloric quantities and supplying the same protein and vitamin intakes (Forbes et al., '46b, '46d), the heat increment or energy expense of utilization of the diet was similarly found to decrease as the fat content of the diet increased.

Each of these investigations consisted of a series of two separate trials, one with greatly increased intakes of 10 of the vitamins. This was done to eliminate the possibility that results were influenced by a suboptimum intake of one or more of the vitamins. However, no significant changes were observed due to the higher level of vitamin intake.

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During the course of this work it became evident that the amount of energy expended in heat production, other than the dynamic effect, varied with the intake of fat and was of importance in explaining the differences in total heat production, particularly among the growing rats.

While this work had not shown any consistent difference in metabolizable energy on the various levels of fat intake, it had shown a marked difference in heat increments where growth was not a factor. Therefore, the question of the heat production which resulted from activity was of interest, and especially its possible relation to the intake of different amounts of fat.

No report dealing directly with this particular subject is known to the present authors. Of interest, however, is the work of Scheer, Dorst, Codie and Soule ('47), who found that the physical capacity of rats increased as the fat content of the diet increased from 0 to 40%. Total activity for the entire day was not measured, but the rats on high fat diets were capable of a more sustained period of excercise than those on low fat rations.

The evaluation of activity in terms of calories presents a very difficult problem. An indirect approach to it was made by measuring the total heat production under conditions of normal cage activity and again with activity restricted to a minimum, all other imposed conditions being the same. The difference was considered to represent the energy expenditure associated with activity. Obviously, heat increment includes energy derived from the "work of digestion" of the food, which is a form of activity but, as such, is of interest in this connection only as it is related to dynamic effect.

The work here reported was done in two parts, the first being a measure of the total heat production, with activity included. in mature male rats fed two distinctly different levels of fat intake, 2% and 30% of the diet. In the previous work (Forbes et al., '46a, '46c) in which the total heat production was determined, growing rats were used; it was de-

sired in the present experiment to eliminate the variable of growth by employing physiologically mature animals.

The second experiment was the direct result of the first and was planned to measure the heat production with and without activity on alternate days, the reason for this being that previous conclusions drawn from work done at different times and on different animals would be more clearly established if completed on the same animals with a minimum of time elapsing between the two determinations.

EXPERIMENT NO. 1

Total heat production, with activity included, of mature rats at different levels of fat intake

In this experiment two groups of 12 male rats each were selected on the basis of uniformity of weight and age and placed in individual cylindrical cages 8 inches in diameter. The animals were approximately 6 months of age at the start and weighed an average of 390 gm. During a preliminary period of one week a constant feed intake of the colony diet was fed at about the same level of energy intake as was planned for the experimental ration to follow. One group of 12 rats was then placed on the low fat diet and the other group was given the high fat diet. The feed intake was maintained constant from this point for the remainder of the experiment, with each animal receiving the same amount of energy and protein each day. After one week of preliminary feeding a 10-day collection of feces and urine was made in metabolism cages which have been used extensively in this laboratory for the separate collection of feces and urine (Swift, Kahlenberg, Voris and Forbes, '34). The one variation made from the method as originally described was that at the beginning of the collection period 100 ml of a preservative solution containing 2 gm of cupric sulfate and 1 gm of sodium fluoride were evaporated to dryness in the crystallizing dish in which the urine was collected. This dish with the preservative was washed out only at the end of the 10-day collection period and not each day.

Following the collection period, the CO_2 production was measured continuously for two days, using respiration cages sufficiently large to accomodate an individual rat cage (Forbes, Swift and Black, '38). The animals therefore had the same freedom of movement during the time when the heat production was being measured as they normally had.

A second period for collection of excreta immediately followed the first test in the respiration chambers, and this was followed by another CO_2 measurement. The diets were then reversed and after the usual preliminary period to establish the animals on the new feed a series of two more excreta collections and CO_2 production periods was instituted. In other words, each rat, with a few exceptions due to refusal of feed, had two periods on the low fat and two on the high fat diet, both for collection of excreta and for determination of the carbon dioxide. The actual number of rats completing each period is shown in table 1. Heat production in all cases was computed by the carbon-nitrogen balance method as described by Forbes, Swift and Black ('38) and as indicated in table 2.

A correction for a CO_2 blank, not considered in the original procedure, was made in this experiment as it was found that a small amount of CO_2 was always obtained in blank runs when excreta were present in the collection dishes in the respiration cages. During the respiration measurements excreta were collected under a layer of lightweight paraffin oil and 100 ml of the copper sulfate-sodium fluoride preservative solution. Although this solution completely covered both the urine and feces as they fell into the dish after being voided, there was apparently some decomposition taking place before the end of the two-day run, with resultant production of CO_2. The amount of CO_2 actually obtained from an average of 18 blank runs with one-day collection of excreta in the respiration chamber was 26 mg.

The diets fed were prepared to be as nearly identical as possible to those used in earlier studies of the relationship between fat and economy of food utilization (Forbes, Swift, James, Bratzler and Black, '46c). The high level of vitamin

intake referred to was provided in all the diets in this and the following paper, in order to avoid any possible influence resulting from the suboptimum intake of an essential nutrient. The feed intake was maintained constant for the entire experiment for all rats on each diet, thus supplying the same energy and protein and essentially the same amount of vitamins and minerals per rat per day.

TABLE 1

Experiment 1. Partition of average daily nitrogen intake per rat

NITROGEN DATA	PERIOD 1		PERIOD 2		PERIOD 3		PERIOD 4	
	Fat in diet		Fat in diet		Fat in diet		Fat in diet	
	2%	30%	2%	30%	2%	30%	2%	30%
	12 animals		11 animals		9 animals		9 animals	
Intake								
mg	542	549	542	549	542	549	542	549
Digested								
mg	491	503	491	504	494	506	493	503
% of intake	90.6	91.6	90.6	91.8	91.2	92.2	91.0	91.6
Urine								
mg	469	479	449	455	433	451	448	459
% of intake	86.5	87.2	82.8	82.9	79.9	82.1	82.7	83.6
Retention								
mg	22	24	42	49	61	55	45	44
% of intake	4.1	4.4	7.7	8.9	11.3	10.0	8.3	8.0

Nitrogen was determined by the standard Kjeldahl procedure and energy by the Emerson bomb calorimeter. Carbon was determined by a wet combustion method similar to that described by Furman ('39), in which the carbon is oxidized to CO_2 by a mixture of sulfuric, phosphoric and chromic acids. The CO_2 evolved was then collected in soda lime contained in glass stoppered U-tubes and weighed.

DISCUSSION OF RESULTS (EXPERIMENT NO. 1)

In table 1 the partition of nitrogen for the 4 periods is presented, showing the intake, excretion and body gain. The average live weights of the rats for periods 1, 2, 3, and 4 were, respectively, 364, 377, 400 and 403 gm for the 2% fat diet, and 362, 383, 392 and 397 gm for the 30% fat diet.

In each of the 4 periods the rats receiving the 2% fat diet excreted more nitrogen in the feces and less in the urine than

TABLE 2

Experiment 1. Intake and partition of daily food energy

FOOD ENERGY DATA	PERIOD 1		PERIOD 2		PERIOD 3		PERIOD 4	
	Fat in diet 2%	30%	Fat in diet 2%	30%	Fat in diet 2%	30%	Fat in diet 2%	30%
	Cal.	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*
Intake	61.52	61.52	61.52	61.52	61.52	61.52	61.52	61.52
Feces	2.95	2.75	2.90	2.80	2.87	2.85	2.98	2.77
Urine	4.03	4.12	3.87	3.92	3.73	3.88	3.85	3.94
Metabolizable	54.54	54.65	54.75	54.80	54.92	54.79	54.69	54.81
Body gain								
As protein	0.77	0.81	1.40	1.66	2.10	1.90	1.56	1.52
As fat	7.94	8.55	6.45	5.92	5.48	6.20	5.53	5.81
Total body gain	8.71	9.36	7.85	7.58	7.58	8.10	7.09	7.33
Heat production [1]	45.83	45.29	46.87	47.23	47.34	46.69	47.60	47.47
Coef. of variation (heat production)	3.6	4.8	4.2	3.5	5.9	3.3	5.5	3.5

[1] Includes normal activity.

did the rats consuming the high fat diet. These differences are insignificant, and the total nitrogen excretion in the feces and urine was essentially the same on both diets.

Table 2 gives a summary of the average daily energy balances for the 4 periods on the high and low fat diets. The high metabolizable energy of both essentially synthetic diets was due to the lack of any bulk or roughage. Slight differences are noted between the energy losses in the feces and urine, but since these are in opposite directions the metabolizable energy values are closely similar.

To determine heat production by the carbon-nitrogen balance method the experimental animals must be thoroughly established on a constant feed intake before any heat measurements can be made that will be thoroughly representative of the conditions imposed. The level of feed intake chosen must not, therefore, be greater than the animals will consume regularly and is always less than on ad libitum feeding. When the rats were first removed from the colony diet and given a weighed amount of the experimental ration, a decrease in weight occurred for several days, some of which was doubtless "fill." After adjustment to a constant amount of the new feed there was a small gain of energy, ranging from 7 to 9 cal. per day throughout the experiment.

In estimating the amount of feed to be given daily at the beginning of the experiment it was known that the theoretical objective of no change in body weight was impossible, but an attempt was made to have the balance on the positive side instead of the negative as losses in body tissue involve a greater loss of heat (dynamic action) than do gains in tissue.

The daily heat productions were similar for both diets in each of the 4 periods, although the trend in three was for the rats on the low fat diet to have a slightly higher heat production than those on the high fat diet.

This uniformity in heat production was somewhat surprising, since the heat production as measured by the body balance procedure in growing rats was greater on the low fat diet than on the high fat diet, with equal energy intakes (Forbes, Swift, James, Bratzler and Black, '46c). The one big difference between these two experiments was in the age of the rats and, consequently, in their physiological status. In the young, rapidly growing animal growth is undoubtedly one of the first, if not the first, demand made upon the energy available to the animal after the needs of vital activities have been satisfied. When the animal has reached maturity, as was the case in the present experiment, the urge to grow has practically ceased and the intake as well as the expenditure of energy was governed by factors other than growth.

In view of the results of this experiment, which show essentially the same heat production as well as metabolizable energy on a high and a low fat intake, and in view of the earlier work in which it was found that the dynamic effect was less on the high fat diet, at least under the experimental conditions imposed, it may be concluded that on high fat diets more heat remains to be dissipated in various forms of activity. It is recognized that this conclusion is drawn from data in two separate experiments conducted at different times and on different animals, but the evidence, as obtained, points to a distinctly greater amount of activity in the rats on the high fat diet.

EXPERIMENT NO. 2

Heat production of mature rats, with and without activity, at different levels of fat intake

The second part of the work reported in this paper originated with the idea that it would further strengthen the data obtained to plan an experiment in which the heat production was measured on consecutive days in the same animals with activity restricted in one case and with normal cage activity in the other. The conclusion drawn above that activity is greater in rats receiving high fat diets than in those on low fat diets is dependent, in part, on results from a study of heat increments (Forbes et al., '46d), and for this reason might possibly be criticized in that it is based on work completed under different conditions and at different times. The technique of combining the entire procedure into one experiment would not only satisfy this criticism but also give additional information on the question of total heat production with unrestricted activity at different levels of fat intake.

Two groups of mature male rats were selected, 10 animals in a group, to be established on rations containing 2 and 30% of fat. The rations were identical with those used earlier and contained the high level of vitamin intake. The rats were also selected from the same group of animals used before but were not all the identical animals. Their age had increased by 6

months and at the time respiration measurements were started they were approximately one year old.

The experimental routine for collection of excreta and care of the animals was essentially the same as that reported in the first part of this paper. The excreta were collected for one period of 9 days' duration in this case and the collection always preceded the respiration measurements.

The total heat production, with activity unrestricted, was measured by the carbon-nitrogen balance method for two complete days, as explained earlier. The heat production with activity restricted was measured for 7 hours by the open-circuit respiratory quotient Haldane procedure with equipment used routinely in this laboratory. In this procedure the rats were confined in half-gallon Mason type jars for respiration chambers and a bright light was placed immediately overhead to keep them quiet. This has proved to be the most satisfactory method of restricting activity which the writers have tried. To eliminate figures for periods when activity was unavoidably high, the CO_2 production was determined hourly and those hours which differed from the average of the final 6 hours by more than $\pm$ 6% were excluded.

An effort was made to standardize conditions in every way possible in order to maintain uniformity of metabolism. These precautions included maintenance of a constant room temperature by thermostatic control at a point slightly above the critical temperature for the rat, and exact regularity in time of feeding. The animals were familiarized with the apparatus by being placed in it for several hours during the preliminary feeding period, the respiration measurements being so arranged that for about half the animals the Haldane method preceded by one day the carbon-nitrogen balance method, while for the others the order was reversed and the Haldane method was used last instead of first.

DISCUSSION OF RESULTS (EXPERIMENT NO. 2)

In table 3 are presented the average daliy intake and balance of nitrogen for the rats on the two diets. The high

digestibility of the nitrogen is apparent again in this experiment, as is the close similarity of the nitrogen in the urine on the two diets. The retention of nitrogen reflects the aim of the feeding schedule, in which an effort was made to approximate the maintenance level of intake yet to have a small positive balance. Retention figures are given mainly to show the status of the animal relative to the plane of nutrition and not so much to demonstrate the efficiency of utilization of this component of the diet. To do this, planes of nutrition other than maintenance would be included.

The significance of the nitrogen utilization figures lies in the close agreement of results between the two diets, indicating that the fat intake did not influence retention of nitrogen.

TABLE 3

Experiment 2. Partition of average daily nitrogen intake per rat

FAT IN DIET	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION
%	mg	mg	% of intake	mg	% of intake	% of intake
2	505	464	91.9	456	90.3	1.6
30	504	468	92.9	464	92.1	0.8

In one previous instance (Forbes et al., '46a) nitrogen retention was in the order of increasing fat content of the diet, but in all subsequent work no difference has been found.

The metabolizable energy values of the daily food, as reported in table 4, show that a somewhat greater amount of metabolizable energy was furnished by the low fat diet. Due to the consistency of these figures the odds that this difference is significant are extremely high, even though the actual difference is small. In previous work using these rations, and in the latest publication in this series of experiments where low protein rations of different fat content were fed (French, Black and Swift, '48), the metabolizable energy has been relatively similar irrespective of the fat content of the diet. The greatest difference observed in any of these experiments

was 2% (Forbes et al., '46c), this difference being in favor of the low fat ration and therefore comparable to the present finding. The significance of these observations must necessarily be considered from its broader aspect and not from the point of view of a single observation. In this respect the fat content of the rations did not consistently affect its metabolizable energy, but when a difference was noted it was in a decreasing order as the fat content of the diet increased. This point emphasizes the need for caution in interpreting small though apparently significant differences in biological work.

Heat production in this experiment as measured with activity either restricted or unrestricted follows closely the pattern of the previous experiments and thereby confirms the results

TABLE 4

Experiment 2. Intake and partition of daily food energy

FAT IN DIET	INTAKE	FECES	URINE	METABOLIZABLE ENERGY	BODY GAIN	HEAT PRODUCTION: With activity included	Coef. of variation	With activity excluded	Coef. of variation
%	Cal.	Cal.	Cal.	Cal.	Cal.	Cal	%	Cal.	%
2	57.67	2.46	3.98	51.23	7.72	43.57	4.0	38.44	6.9
30	57.67	3.66	4.02	49.99	7.97	42.06	2.9	36.15	7.1

obtained, especially as reported in the first part of this paper. This is an important point and indicates that a number of variable factors such as different animals, time of year, experimental routine, et cetera, did not materially influence the results obtained. Through use of the technique of having the respiration measurements made with the same animals on consecutive days there can be little doubt of the part that activity plays in total heat production. It is recognized that one reasonable possibility remains which could influence the respiration measurements obtained with the Haldane method, since the period of observation does not extend over a full day and any change in metabolism during a time other than when the measurements were being made would not be re-

flected in the final result. No claim ignoring this possibility has been made in any of the previous papers and, in fact, it has been specifically stated that the conclusions drawn applied only to the manner of experimentation employed.

With this general idea in mind, some preliminary work was initiated in this experiment which indicates the possible existence of well-defined changes in the curve of total heat production over a 24-hour period. These fluctuations in daily heat production, both with and without activity, have been investigated in detail and will be reported in a later paper.

The observed difference in total heat production between the two groups of animals of 1.51 Cal. per day (table 4) is the largest yet obtained. Although the odds that this difference is significant are 38 to 1, little emphasis is placed on it since the work taken as a whole has shown no significant difference in heat production between the two groups of mature rats when determined by the carbon-nitrogen balance method with activity unrestricted.

Contrasted with the similarity in heat production observed when activity was uncontrolled is the greater difference when activity was limited. In this experiment it is noted that the difference in average daily heat production between the two diets was 2.29 Cal., or, expressed in another way, the rats on the low fat ration produced 6.3% more heat than those on the high fat ration. The odds that this difference is significant are 555 to 1. This finding confirms previous work when the Haldane procedure was used, the values reported in the two earlier papers showing that the rats on the low level of fat intake produced 10.7 and 8.7% more heat than those on the high fat ration at the maintenance level of intake (Forbes et. al., '46b, '46d). This comparison can be made only on this level, since the supermaintenance levels, as used with the maintenance levels in the determination of heat increments, represent a much greater intake of food than was given in the course of the present work.

In conclusion it may be said that the total heat production with activity unlimited was not affected to any appreciable

extent by the level of fat intake, but the heat production as determined with activity restricted was markedly affected. This latter finding harmonizes with our previous observations, and since the metabolizable energy is essentially the same on either diet the remainder of the energy balance or energy expended on activity must therefore be greater on the high fat diet, varying reciprocally with the heat increment.

SUMMARY

In two separate experiments a study was made of the relation of dietary fat to the amount of spontaneous voluntary activity in the mature albino rat.

Due to the difficulty of measuring activity directly, it was estimated as the difference between the total heat production measured with and without activity.

In the first experiment the total heat production of two groups of 12 rats each was measured by the carbon-nitrogen balance method for 4 periods, each of two days' duration, with the animals being permitted normal freedom of movement during the respiration measurement. Equicaloric diets of either low (2%) or high (30%) fat content and containing the same amount of protein, vitamins and minerals were fed, with reversal of diets being made when the experiment was half completed.

It was found that the heat production, as well as the utilization of nitrogen, was not influenced appreciably by the level of fat in the diet. As the heat increment had previously been found to be greater on a low fat diet, it was concluded that the energy expended in all forms of activity must be greater in animals on a high fat diet, varying in a reciprocal manner with the heat increment.

To substantiate this claim relative to the amount of activity, a second experiment using the same animals was conducted in which the heat production was measured on consecutive days, first with activity unlimited and then with it limited. For the latter estimation the Haldane procedure was used, with the respiratory measurement lasting 7 hours.

The results obtained under both conditions of experimentation agree closely, in principle, with former observations showing approximately the same total heat production on a high fat diet when activity was permitted but a lower heat production when activity was limited.

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FURTHER EXPERIMENTS ON THE RELATION OF FAT TO ECONOMY OF FOOD UTILIZATION

V. FLUCTUATIONS IN CURVE OF DAILY HEAT PRODUCTION [1]

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ONE FIGURE

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In the previous paper evidence was presented showing that the over-all activity of experimental animals increased as the fat content of the diet increased (Black, French and Swift, '49). This evidence also indicated that this increase in activity was related in a reciprocal manner to the heat increment. These findings were applicable to mature albino rats only, since it had been found in earlier work that the heat production of growing rats was less on a high fat diet than on a low fat diet (Forbes et al., '46a, '46c). This difference in results is attributed to the physiological status of the animal, as the demand for growth in the young animal is undoubtedly great. In the mature animal this demand to grow does not exist and the energy intake and expenditure are in a state of approximate equilibrium.

Evidence of a preliminary nature obtained in this work indicated a marked variation in heat production at different times of the day. Since the rat is a nocturnal animal, most of its activity would be expected at night or during the time when the laboratory was dark. It is therefore of interest to know something about this fluctuation in heat production

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throughout the 24-hour cycle with activity unrestricted, as well as during the shorter periods which are used in the measurement of heat increments in which activity is limited. It seemed possible that the heat increment, measured under definite imposed conditions and for a part of the day only, might not represent the total daily dynamic effect actually produced under conditions of normal activity. Although considerable thought has been given this problem in the past, it appeared advisable to attack it again in connection with the present study of fat metabolism.

The existence of a diurnal metabolic rhythm in animals has been claimed by many investigators, with fluctuations in metabolism which are dependent mainly upon light cycles. Irritability, activity, muscular tonus, feeding times, et cetera, are factors which are influenced by light intensity, and these in turn alter the metabolic rate. The information on this subject appearing in the literature has been summarized by Brody ('45).

For the rat, Herring and Brody ('38) reported a diurnal metabolic rhythm with maximum variations of 25–30%. This evidence further indicates the desirability of investigating fluctuations in metabolism in relation to the problem under consideration.

EXPERIMENT NO. 1

Relation of fat to total heat production in the rat during 24-hour cycle with activity unrestricted

The specific purpose of the present experiment was to determine the changes in metabolic activity in the rat during a 24-hour cycle when diets containing either a low (2%) or high (30%) fat content were fed. The diets were compounded and fed so as to supply not only equal quantities of protein and energy but also essentially the same amounts of minerals and vitamins. The composition of the diets was practically the same as in previous studies on fat metabolism in this laboratory, and is given in tables 1 and 2 of an earlier paper by Forbes et al. ('46c). There was a slight difference in the

amounts of the protein and carbohydrate mixtures used, depending on the chemical composition of the ingredients in the mixtures. It is to be noted that the rations contained high levels of vitamins so that there would be no possibility of a suboptimum intake of any of these essential nutrients.

The subjects were 24 mature male rats, 16 to 17 months of age, with 12 rats in each of two groups. One group was fed the low fat diet, the other the high fat diet. The rats were selected from the same group of animals used in the previous work on this subject but were not necessarily the identical animals (Black, French and Swift, '49).

In an effort to have the same dynamic effect of the feed manifest on the curve of heat production during the day as at night, the daily allotment of feed was divided into two equal portions and fed exactly 12 hours apart, at 8 A.M. and 8 P.M.

The animals were thoroughly established on the experimental routine in the usual preliminary feeding period prior to a 10-day period for collection of feces and urine. The procedure followed for this collection was the same as that used normally in this laboratory and described in detail by Swift, Kahlenberg, Voris and Forbes ('34), except that a solution of cupric sulfate and sodium fluoride was used as a preservative in the crystallizing dish in which the urine was collected. The dish was washed out only at the end of the collection period.

The respiration measurements were conducted for two days following the collection period, this being done continuously for the 48-hour period in respiration chambers of sufficient size to accommodate the laboratory cage (Forbes, Swift and Black, '38). In order to determine the changes in metabolism throughout this time, ventilation of the chambers was stopped long enough every three hours to weigh the absorption tubes. This required only about three to 4 minutes on the average and was of such short duration that it did not permit any appreciable increase in CO_2 concentration in the respiration chamber. Any accumulation of CO_2 was thoroughly removed during the ensuing three-hour period. As the tubes from all

6 chambers could not be weighed simultaneously, it was necessary to stagger the time of weighings. This was done at 10-minute intervals, starting on the hour for the first cage. The actual times throughout the day when the weighings were made are the points indicated in figure 1, beginning at 1 P.M. and continuing every three hours thereafter.

Heat production was computed by the carbon-nitrogen balance procedure for each three-hour period, as well as the total for each of the two complete days. The method is described in detail by Forbes, Swift and Black ('38). The information on excretion of urinary and fecal carbon and nitrogen was computed from data comprising the average level of excretion for the 10-day collection period prior to the respiration measurements. For the three-hour periods three twenty-fourths of the average daily excretion was used, as it would have been impossible to make separate collections for such short periods.

Normal voluntary activity was permitted during the respiration measurements, the same cages being used during this metabolism test as during the rest of the experiment.

DISCUSSION OF RESULTS (EXPERIMENT NO. 1)

Table 1 presents the average daily intake and excretion of nitrogen. The values are in very close agreement with those reported previously using these rations (Black, French and Swift, '49), and again show that the level of fat did not influence the excretion or utilization of nitrogen under the circumstances imposed. The evidence indicates that the original objective, to feed at the maintenance level, was attained.

The difference of 17 mg in the daily intakes of nitrogen is due to unavoidable slight errors involved in analyzing and compositing the ration components while seeking to make the rations contain equal amounts of nitrogen and energy per unit of feed.

The data on daily intake and partition of energy, given in table 2, depict results which are quite similar to those obtained in previous work using this procedure (Black, French and

Swift, '49). Although the main objective was to show the hourly fluctuations in heat production, it was considered advisable to present the daily values as well since these figures relate the different experiments more closely. The daily heat production of the two groups of animals varies somewhat more than in any previous experiments but the variation is in the same direction as formerly, with the rats on the low fat

TABLE 1

Partition of average daily nitrogen intake per rat

FAT IN DIET	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION
			% of intake		*% of intake*	*% of intake*
%	*mg*	*mg*		*mg*		
2	512	471	92.0	462	90.2	1.8
30	529	495	93.6	485	91.7	1.9

TABLE 2

Intake and partition of daily food energy

FAT IN DIET	INTAKE	FECES	URINE	METABOLIZABLE ENERGY	BODY GAIN	HEAT PRODUCTION	
						Amount	Coefficient of variation
%	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	*Cal.*	%
2	57.43	2.50	4.04	50.89	5.21	45.68	4.93
30	57.42	2.15	4.25	51.02	7.35	43.67	2.43

diets producing more heat. The odds that this difference is not due to chance alone are 205 to 1, whereas the greatest significance obtained in any of our previous work using similar diets and techniques was characterized by odds of 38 to 1 (Black, French and Swift, '49). The tendency of a high fat diet to produce less heat than an equicaloric diet of low fat content is not as pronounced when the experimental conditions involve mature rats with unrestricted activity as it is when activity is restricted or young animals are used.

The curve of heat production extending continuously for a period of 48 hours and divided into three-hour intervals is presented in figure 1. It is apparent that the characteristic curves for the two diets showing the effects of the time of day and of the ingestion of food are definitely different in certain respects. On the low fat diet there was an increase in heat production after food was given either in the morning or evening, but the rise was much less during the day than at night. The increase during the morning seems to be largely dynamic effect, since it did not start until food was given,

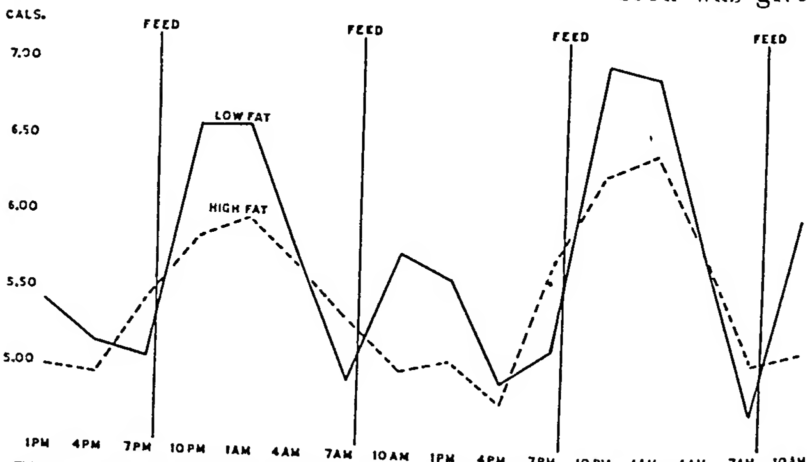

Fig. 1 Curves of heat production of rats on diets of high or low fat content.

while in the evening the increase started slowly between 4 P.M. and 7 P.M. This is obviously due to the activity of the rats, a fact noticeable to the observer during the hour prior to feeding. The peak of the dynamic effect and whatever extra activity there was during and immediately after eating the low fat diet was always reached within two hours after consuming the food, with a decrease in heat starting thereafter. This decrease was gradual at night, and even at 4 A.M., metabolic activity was greater than at any other time during the day.

The difference between the amount of activity during the day and night is clearly demonstrated by the animals on either

diet. There appears to be little activity by the rats on the high fat diet during the day, but after 4 P.M. an increase is evident which continues throughout the night.

The ingestion of the high fat diet had little demonstrable effect on the metabolic activity. This is in harmony with the results of the study of the dynamic effects of these diets (Forbes et al., '46b, '46d), in which it was found that the high fat diet had a much lower dynamic effect than did the low fat diet. At first glance the statement above does not appear to be entirely correct, since the heat production of the high fat rats rose to a great extent during the night. Part of the rise might be attributed to the heat increment of the evening feed, but this is contraindicated by the feeble rise in heat production following the morning feeding.

It is of particular interest and concern that the heat production of the rats on the high fat diet was greater at two specific times during the day than in the rats on the low fat diet, these times being between 4 and 7 P.M. and 4 and 7 A.M. Also the magnitude of the difference in metabolism between the two groups was greatest from 10 A.M. to 1 P.M., or during the time of day which had previously served as a basis for the study of heat increments. In other words, the conditions arbitrarily chosen for these experiments were those most likely to give a distinctly higher heat increment on a low fat diet. This problem has been investigated and is to be reported on in the second part of this paper.

Differences in gains of energy are accompanied by approximately equal and opposite differences in heat production (table 2). Results of previous work have also invariably involved a greater body gain of energy with accompanying lesser heat production on high vs. low fat diets. It is of interest to note that digestibility, metabolizability, dynamic effect or activity were not the factors directly related to gain of energy; only heat production has been so associated.

The metabolic rhythm noted in this study agrees to a considerable extent with the work of Herring and Brody ('38), in which they found fluctuations in the metabolism of the

rat varying from 25–30%. They measured metabolism by the Haldane procedure and doubtless the activity was restricted to a greater extent than in the work reported here, in which a rise of 40% in heat production was noted in the animals on the low fat diet. The influence of the type of diet on the daily metabolic fluctuation is apparent from the present study.

This work again points to the importance of considering the use of relatively long periods in the study of energy metabolism, as short periods may give no hint of factors prevailing at some other time of the day or at some point of a cycle other than the one in which the study is made.

EXPERIMENT 2

Relation of fat to heat production in the rat with activity restricted

In view of the results reported in the first part of this paper, showing the fluctuations in heat production at different times of the day in rats that were fed diets of varying fat content and permitted normal freedom of movement in the cage, it appeared of equal importance to learn what variation there might be in the metabolism when normal voluntary activity was restricted, for it is under this condition that the heat increment or dynamic effect has been determined.

This laboratory has been concerned with the problem for many years and has given much thought to it in planning experiments in which the heat measured was not to include that due to activity. The present experiment is by no means the first time the problem has been considered.

Although most investigators have for convenience used the daylight hours to measure the metabolic activity of rats, it has not been proved that this procedure is necessarily the most reliable. The present experiment was designed to produce further information on this subject, especially as the results might be influenced by the fat content of the diet.

The general routine of the experiment was the same as that outlined in an earlier paper (Forbes et al., '46d) with one ex-

ception, this being that the time of respiration measurements was shifted to begin about 1 P.M. and extend to 7:30 P.M. instead of starting at 8 A.M. and continuing to 3:30 P.M. This was done so as to include in the period of observation one of the times of day during which the heat production of the high fat rats was higher than the low fat rats under conditions of uncontrolled activity (fig. 1). This choice of time would be the least favorable to give a high heat increment on a low fat diet, a result previously reported.

Heat productions were determined by the Haldane respiratory quotient procedure as in previous similar experiments. Activity was limited by the type of respiration chamber as well as by a bright light placed over the chamber. To make possible the elimination of any intervals when activity was unavoidably high, the CO_2 production was determined hourly, and those hourly weights of CO_2 which differed from the average for the final 6 hours by more than $\pm$ 6% were excluded. The heat increment was determined as the difference in heat production of the same animals on maintenance and supermaintenance quantities of the same diets.

The subjects were two groups of 12 essentially mature albino rats about 6 months of age.

The diets were composed as previously reported (Forbes et al., '46c), one containing 2% fat, the other 30% fat. The rats on the 2% fat diet received 11 and 16 gm each of food at the two planes of nutrition, whereas the rats on the 30% fat diet received smaller quantities but isocaloric equivalents for the two planes of nutrition.

The schedule of experimentation was so arranged for each rat that a two-day maintenance respiration period began immediately after an 8-day maintenance excreta collection period.

Then followed a 5½ day preparatory feeding on the supermaintenance plane, a two-day period for respiration measurement on the supermaintenance feeding, and an 8-day excreta collection period on the same intake. This order of experimentation was followed so as to have the respiration periods

at the two planes of nutrition as close together as possible. In this way the weight and age of the animals were essentially the same at both planes of nutrition and correction for changes in live weight was unnecessary.

DISCUSSION OF RESULTS (EXPERIMENT NO. 2)

The digestibility of nitrogen (table 3) was essentially the same at both planes of nutrition, though slightly higher on the high fat diet at both levels of intake. No significant difference was found in retention of nitrogen, an observation consistent with results from previous experiments using these diets.

TABLE 3

Utilization of daily nitrogen

FAT IN DIET	PLANE OF NUTRITION	NITROGEN INTAKE	NITROGEN DIGESTED		NITROGEN OF URINE		NITROGEN RETENTION	
%		*mg*	*mg*	*% of intake*	*mg*	*% of intake*	*mg*	*% of intake*
2	Maintenance	392	364	92.9	387	98.7	—23	—5.9
30	Maintenance	394	371	94.2	395	100.3	—24	—6.1
2	Supermaintenance	570	530	93.0	485	85.1	45	7.9
30	Supermaintenance	573	538	93.9	490	85.5	49	8.6

The data on intake and partition of daily food energy (table 4), show little difference between the two diets in the energy of the urine or in metabolizable energy and only a slightly lesser amount of fecal energy on the higher level of fat intake. The percentage of metabolizable energy appearing as heat is less at both planes of nutrition on the high fat diet, indicating that the fat increased efficiency of utilization of the metabolizable energy in this work as in earlier experiments. The odds that the metabolizable energy of the 30% fat diet was more efficiently utilized than that of the 2% fat diet are 9600 to 1 at the supermaintenance plane of nutrition and 30 to 1 at the maintenance plane.

The heat productions as well as the heat increments (table 5) diminished markedly at the higher level of fat intake. The heat increments reported in an earlier paper were 6.16 and 3.29 Cal. for the 2 and 30% fat diets, respectively (Forbes et al., '46d). This represents a decrease of 47% from the low to the high fat. The same values in table 5 show a difference of

TABLE 4

Partition of daily food energy

FAT IN DIET	PLANE OF NUTRITION	INTAKE	FECES	URINE	METABOLIZABLE ENERGY		HEAT PRODUCTION		METABOLIZABLE ENERGY AS HEAT
					Amount	Coeff. var.	Amount	Coeff. var.	
%		*Cal.*	*Cal.*	*Cal.*	*Cal.*	%	*Cal.*	%	%
2	Maintenance	45.31	1.90	3.15	40.25	0.4	28.62	7.5	71
30	Maintenance	45.20	1.68	3.22	40.30	0.3	26.82	6.4	67
2	Supermaintenance	65.90	2.57	4.51	58.83	0.2	34.20	5.8	58
30	Supermaintenance	65.74	2.48	4.57	58.69	0.4	31.25	4.8	53

TABLE 5

Quantities, increments, and sources of average daily heat production

FAT IN DIET	PLANE OF NUTRITION	TOTAL HEAT AND HEAT INCREMENT	NON-PROTEIN R. Q.	SOURCE OF HEAT PRODUCTION			
				Protein	Carbohydrate	Fat	Fat synthesis
%		*Cal.*		*Cal.*	*Cal.*	*Cal.*	*Cal.*
2	Supermaintenance	34.20	1.17	12.85	20.58	0.00	0.77
2	Maintenance	28.62	1.08	10.26	18.06	0.00	0.30
2	Heat increment	5.58		2.59	2.52		0.47
30	Supermaintenance	31.25	0.89	12.98	11.73	6.54	0.00
30	Maintenance	26.82	0.85	10.47	8.29	8.06	0.00
30	Heat increment	4.43		2.51	3.44		0.00

21%. Some of the variation between the two experiments may have been due to the time after the last feeding when the heat increment was measured. In the first experiment the respiration measurement was started shortly after feeding, while in the present experiment it was not begun until 5 hours after feeding.

The main reason for the difference in heat increments between the two experiments is attributed, however, to the fluctuations in the curve of total heat production (fig. 1). The control of activity in the Haldane procedure is not perfect and it is conceivable that there might be sufficient movement in the animals, late in the afternoon, approximately to equalize the heat productions on the two diets and thus produce the same heat increments. It is gratifying that this did not occur, and even though the magnitude of the difference between heat increments was not as large as previously reported, the fundamental findings were satisfactorily verified. A knowledge of the fluctuations in metabolism during the 24-hour day is most useful in planning experimental work.

SUMMARY

Fluctuations in the curves of heat production for the 24-hour day for albino rats receiving equicaloric diets containing either 2 or 30% fat have been studied, using 12 rats on each diet. The heat production was measured by the carbon-nitrogen balance procedure at intervals of three hours each.

Characteristic differences in the curves of heat production for the two diets were observed. The influence of activity at different times of the day is clearly evident, as is the difference in dynamic effect of the diets.

Although fluctuations in metabolism were great among three-hour periods, the total heat for the day compared favorably with earlier results.

A further study was also made of the dynamic effect of the diets to determine whether activity had been a serious factor in these measurements. By choosing a time of day in which to make these determinations least likely to support

previous contentions as judged by the curve of total heat production, it was found that the high fat diet resulted in a much lower heat increment than the low fat diet, again confirming earlier findings.

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THE COMPARATIVE ENHANCEMENT OF THE BASAL METABOLISM AND OF THE ENDOGENOUS NITROGEN METABOLISM OF ALBINO RATS IN EXPERIMENTAL HYPERTHYROIDISM

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The demonstration by Terroine and Sorg-Matter ('37) and by Smuts ('35) that there is a relationship approaching constancy between the minimum endogenous nitrogen metabolism and the basal metabolism of energy among young adult animals of many species is a most important contribution to science, with important practical applications. Since the endogenous loss of nitrogen in the urine is the predominant component among the factors determining the maintenance requirement for protein, the constancy of this ratio among adult animals means that the maintenance requirement for protein varies with body size in a manner similar to the basal metabolism, i.e., in proportion to body surface (or to some fractional power of the body weight), rather than to body weight itself as is quite commonly supposed. Evidence, direct and auxiliary, supporting this principle has been cited by Smuts. In later experiments he has shown that the endogenous nitrogen metabolism of sheep (Smuts and Marais, '39) and of pigs (Du Toit and Smuts, '41) varies with body weight in much the same fashion as the basal metabolism.

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Smuts' ('35) experiments on mice, rats, guinea pigs, rabbits and pigs revealed the remarkably constant ratio of endogenous urinary nitrogen to basal heat of 2 mg per Cal. for adult, or near adult, animals, mostly males. Ratios recently reported for adult human subjects are considerably lower than this, averaging 1.42 mg per Cal. for 9 women (Bricker, Mitchell and Kinsman, '45), 1.41 mg per Cal. for 5 men (Murlin et al., '46) and 1.19 mg per Cal. for 6 women and 1.32 mg per Cal. for 7 men (Hawley et al., '48). Bricker, Mitchell and Kinsman discuss the possibility of a sex difference affecting the ratio and cite supporting evidence from other investigations. The University of Rochester group are frankly skeptical of the existence of any significant relationship between the endogenous nitrogen of the urine and the basal heat. In the words of the authors of the 1946 report referred to above: "It seems incongruous that one phase of (protein) maintenance metabolism which cannot be attained inside a month should be so definitely ('rigorously' is the term used by Terroine and Sorg-Matter) proportional to another phase (energy) of maintenance metabolism which can be attained in 15 hours." It should be noted that the statement by these workers concerning the time required to attain the minimum endogenous metabolism is contrary to their own experience. It may be noted further that the two experiments reported from the Murlin group were carried out in the summer months, the later one during "severely hot, humid weather." The losses of nitrogen in the sweat were not measured, were probably considerable, and quite probably diminished the urinary nitrogen on a nitrogen-free diet, since urea and ammonia account for over 50% of the nitrogen of sweat (Cuthbertson and Guthrie, '31). Sweat also contains creatinine (Mosher, '33; Mickelsen and Keys, '43), the urinary constituent uniquely characteristic of the endogenous catabolism. We have found that the summertime is a poor season in which to carry out accurate nitrogen, calcium, iron or iodine balance experiments by the usual methods on humans, because of the considerable losses of these elements in the sweat.

Nevertheless, experimental work subsequent to Smuts' report indicates clearly that the ratio of the minimum endogenous urinary nitrogen to basal heat is subject to disturbance in animals of different ages (Ashworth, '35; Ashworth and Cowgill, '38a; Treichler, '39), after various periods of body nitrogen depletion (Murlin et al., '46), and, in the rat at least, by the plane of nutrition (Treichler and Mitchell, '41). In hypophysectomized and thyroidectomized rats, according to Ashworth and Cowgill ('38b), the basal metabolism is depressed with no constant or appreciable effect on the endogenous urinary nitrogen: hence, the ratio is raised. However, the authors question whether the minimum endogenous level of urinary nitrogen had been reached in these experiments, especially in the thyroidectomized animals. In the experience of Nagornyi and Golubitskaya ('41), thyroidectomy lowers the endogenous nitrogen metabolism of rats of various ages.

The purpose of the experiments to be reported in this paper was to determine whether experimental hyperthyroidism in the rat, a condition known to elevate the basal metabolism readily, would elevate the rate of the endogenous nitrogen catabolism and in the same proportion. Hyperthyroidism has been shown to stimulate the total metabolism of nitrogen in the rat (Terroine and Babad, '39; Sure et al., '41), in the sheep (Blaxter, '48), and in the child (Johnston and Maroney, '39). On the assumption that iodinated casein does not disturb the ultilization of dietary protein, Blaxter ('48) estimates that the endogenous metabolism of nitrogen is markedly increased by this thyroxine preparation. In hyperthyroidism, the creatine output in the urine increases greatly, as shown by many authors, with either no effect on or a depression in the creatinine output. Although in the normal child there is a close relationship between the urinary creatinine and the basal metabolism, in the hyperthyroid child the creatinine output is not increased, in the experience of Talbot and his co-workers ('39).

The results of the experiments to be reported below demonstrate that experimental hyperthyroidism in adult rats, induced by feeding iodinated casein, augments both basal metabolism and endogenous nitrogen metabolism. For the dosages used, the two phases of the maintenance metabolism increase in a parallel fashion so that the ratio between the two is not appreciably disturbed.

TABLE 1

Plan of experiments, with food intakes and body weight changes of rats

PERIOD NO.	RATION	PERIOD FED	FOOD INTAKE	GAIN IN BODY WT.	DETERMINATIONS MADE
		days	*gm/day*	*gm/day*	
		Normal period			
I	Standard	42	13	+ 1.3	Basal heat
II	Low-N	17	16	+ 1.0	Urinary N
III	Standard	10	16	+ 1.6	Basal heat
		Hyperthyroid period [1]			
IV	Standard	14	16	— 0.3	Basal heat
V	Standard	10	19	0.0	Basal heat
VI	Low-N	16	19	— 1.5	Urinary N

[1] Fed 45 mg "Protamone" (iodinated casein) per rat per day. This dosage of protamone was found to increase the basal metabolism by about 70% in an exploratory experiment using identical rats.

MATERIALS AND METHODS

Plan of experiment

The experiments were carried out upon 7 adult male albino rats. They ranged in weight from 200 to 300 gm. Observations were made under normal conditions in periods I, II, and III (table 1) and after inducing hyperthyroidism by feeding iodinated casein [2] in periods IV, V and VI.

A basal metabolism measurement was made in periods I and III upon each of the 7 rats under the normal regime. Since it has been shown by Hamilton ('37, '39) and by Treich-

[2] Protamone obtained from the Cerophyl Laboratories, Inc. ,Kansas City, Missouri, through the courtesy of Dr. W. R. Graham, Jr.

ler and Mitchell ('41) that the basal metabolism of rats is markedly influenced by the previous caloric intake, all basal metabolism determinations were made after feeding the rats a constant amount of the experimental ration for about three weeks. In period II the endogenous urinary nitrogen was determined in a 7-day experiment following an adjustment period of the same length.

After the feeding of iodinated casein was started in period IV, several basal metabolism measurements were made on 4 rats to find out when the basal metabolism reaches a constant high level as a result of feeding the iodinated protein. Two or more basal metabolism tests were made on each of the 7 rats after the basal heat production reached its plateau. Since a slow loss of body weight was observed on iodinated casein feeding in period IV, the food intake in periods V and VI was raised from 16 to 19 gm per day per rat. The endogenous nitrogen was determined in period VI.

Basal metabolism

The basal metabolism of the rats was determined by the gravimetric method of Haldane (1892) as modified by Mitchell and Carman ('26a). The respiratory quotient was determined in experiments lasting two to three hours. Since the animals exhibited occasional activity during this period, the basal production of carbon dioxide was determined in shorter periods (about 30 min.) of complete muscular repose. The rats were fasted for at least 17 hours before the basal measurements were made. The temperature inside the respiration chamber was always within the range of thermal neutrality (28–33°C.) for fasting rats as observed by Swift ('44). In calculating the basal heat production, protein metabolism was not considered and total respiratory quotients were assumed to represent the non-protein respiratory quotients. The error introduced by such simplification rarely exceeds 2% (Smuts, '35, p. 412).

Experimental diets

Two experimental diets were employed (table 2); one, the "standard" diet, was used during all times except when the minimum endogenous urinary nitrogen was to be determined, and the other, the low-nitrogen diet, during periods II and VI. The former ration contained 9.7% and the latter 3.0% of whole egg protein. All rations were fed in amounts to maintain approximately the body weights of the rats.

TABLE 2

Percentage composition of experimental diets

INGREDIENTS	STANDARD RATION	LOW-N RATION
	%	%
Dried ether-extracted whole egg	13.0	4.0
Wood flock [1]	2.0	2.0
Amidex (dextrin)	31.0	32.0
Vitaminized starch [2]	5.0	5.0
Cerelose (glucose)	26.0	34.0
Sucrose	10.0	10.0
Salt mixture 446 [3]	4.0	4.0
Wheat germ oil	0.5	0.5
Cod liver oil	1.5	1.5
Lard	6.0	6.0
Sodium chloride	1.0	1.0
Per cent protein	9.7	3.0

[1] Obtained from Brown Company, Portland, Maine.

[2] Containing all of the known members of the B-complex execept biotin and folic acid.

[3] See Spector ('48).

The endogenous urinary nitrogen excretion

Mitchell and Carman ('26b, p. 200) have shown that whole egg protein at a level of 3 or 4% in the diet, while maintaining the appetite better, does not affect appreciably the output of urinary nirogen on a nitrogen-free diet. The collections of urine were made daily by the methods employed in this laboratory, the adequacy of which has been satisfactorily demonstrated (Smuts, '35, p. 416–17).

Methods of analysis

All urine collections were analyzed for total nitrogen by the Kjeldahl method, using mercury and potassium sulfate in the digestion mixture. Preformed creatinine was determined by the method described by Hawk, Oser and Summerson ('47). For total creatinine, a modification of the Folin method (Mitchell and Carman, '26b) was used; creatine was calculated by difference.

TABLE 3

Summary of metabolism data on the rats in normal periods

RAT NO.	BASAL HEAT PRODUCTION [1]	RESULTS OBTAINED IN PERIOD II			RATIO OF ENDOGENOUS N TO BASAL HEAT
		Gain in body wt.	Endogenous urinary nitrogen		
	cal./m²/day	*gm/day*	*mg/day*	*mg/m²/day* [2]	*mg/cal.*
3	953	+ 0.4	58.9	1614	1.69
4	946	+ 0.5	48.9	1385	1.46
6	786	+ 1.6	42.0	1138	1.45
7	873	+ 1.8	42.9	1205	1.38
8	908	+ 0.5	57.4	1612	1.77
9	927	+ 0.9	57.4	1568	1.69
10	943	+ 1.4	43.7	1190	1.26
Average	905			1387	1.53

[1] Data obtained in period III.
[2] See footnote 1, table 6.

EXPERIMENTAL RESULTS

The metabolic observations on the rats prior to the induction of hyperthyroidism are summarized in table 3. The minimum endogenous urinary nitrogen averaged 1387 mg and the basal metabolism 905 cal. per m² per day. The average ratio between the two is 1.53 mg per cal. This ratio is lower than that reported by Smuts ('35), i.e., 1.99, or that reported by Treichler and Mitchell ('41), i.e., 1.95 for adult rats on maintenance nutrition. The discrepancy may be due in part to the fact that the rats in period III, during which the basal

metabolism was measured, were receiving considerably more than a maintenance ration in anticipation of the higher energy requirements during the hyperthyroid condition that was to be imposed later. During this period they were gaining 1.6 gm per day on the average (table 1). On surfeit feeding, Treichler and Mitchell secured an average of 1.66 for adult male rats.

TABLE 4

Increase in the basal metabolism of the rats after different periods of feeding iodinated casein[1]

RAT NO.	PERIOD ON PROTAMONE	BASAL HEAT PRODUCTION				INCREASE IN HEAT PRODUCTION
		Normal value[2]	R.Q.	Hyperthyroid values	R.Q.	
	days	*cal./m²/day*		*cal./m²/day*		%
4	10	916	0.72	1631	0.74	72
	13			1723	0.72	82
	21			1622	0.76	71
	26			1697	0.74	79
	29			1657	0.74	75
6	9	786	0.73	1349	0.73	71
	23			1330	0.77	69
	28			1296	0.73	65
7	10	873	0.74	1512	0.75	73
	15			1865	0.75	114
	20			1916	0.78	119
8	5	908	0.70	1271	0.71	39
	18			1639	0.71	80
	22			1618	0.72	78

[1] "Protamone."
[2] Values obtained in period III.

In order to determine the time required for the rats to adjust themselves to the selected dosage of protamone (45 mg per rat per day), the basal heat production of 4 of the rats was determined from time to time for a period of three to 4 weeks after dosage started. The values obtained are presented in table 4. It appears that the basal metabolism is approximately stabilized after about three weeks of dosage.

TABLE 5

Metabolism data on the hyperthyroid rats

RAT NUMBER	BASAL HEAT PRODUCTION		CHANGE IN BODY WEIGHT	RESULTS OBTAINED IN PERIOD VI: Endogenous urinary N			RATIO OF ENDOGENOUS N TO BASAL HEAT	TOTAL CREATININE N OF ENDOGENOUS N	CREATINE FRACTION OF TOTAL CREATININE
		Increase over normal				Increase over normal			
	cal./m²/day	%	*gm/day*	*mg/day*	*mg/m²/day*	%	*mg/cal.*	%	%
3	1748	83	—1.6	95.5	2652	61	1.52		37
4	1675	77	—0.3	98.6	2961	114	1.77	6.5	25
6	1313	67	+0.1	97.0	2493	119	1.90	7.1	32
7	1890	116	—0.4	85.7	2498	107	1.32	5.8	27
8	1628	79	—0.4	88.7	2609	62	1.60	7.1	35
9	1731	86	—0.8	91.3	2564	63	1.48	5.6	22
10	1753	86	—1.1	94.1	2536	113	1.45	6.6	23
Averages	1677	85			2616	92	1.58	6.5	29

metabolism was measured, were receiving considerably more than a maintenance ration in anticipation of the higher energy requirements during the hyperthyroid condition that was to be imposed later. During this period they were gaining 1.6 gm per day on the average (table 1). On surfeit feeding, Treichler and Mitchell secured an average of 1.66 for adult male rats.

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Increase in the basal metabolism of the rats after different periods of feeding iodinated casein [1]

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		Normal value [2]	R.Q.	Hyperthyroid values	R.Q.	
	days	*cal./m²/day*		*cal./m²/day*		%
4	10	946	0.72	1631	0.74	72
	13			1723	0.72	82
	21			1622	0.76	71
	26			1697	0.74	79
	29			1657	0.74	75
6	9	786	0.73	1349	0.73	71
	23			1330	0.77	69
	28			1296	0.73	65
7	10	873	0.74	1512	0.75	73
	15			1865	0.75	114
	20			1916	0.78	119
8	5	908	0.70	1271	0.71	39
	18			1639	0.71	80
	22			1618	0.72	78

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In order to determine the time required for the rats to adjust themselves to the selected dosage of protamone (45 mg per rat per day), the basal heat production of 4 of the rats was determined from time to time for a period of three to 4 weeks after dosage started. The values obtained are presented in table 4. It appears that the basal metabolism is approximately stabilized after about three weeks of dosage.

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		Increase over normal				Increase over normal			
	cal./m²/day	%	*gm/day*	*mg/day*	*mg/m²/day*	%	*mg/cal.*	%	%
3	1748	83	—1.6	95.5	2652	61	1.52		37
4	1675	77	—0.3	98.6	2961	114	1.77	6.5	25
6	1313	67	+0.1	97.0	2493	119	1.90	7.4	32
7	1890	116	—0.4	85.7	2498	107	1.32	5.8	27
8	1628	79	—0.4	88.7	2609	62	1.60	7.1	35
9	1731	86	—0.8	91.3	2564	63	1.48	5.6	22
10	1753	86	—1.1	94.1	2536	113	1.45	6.6	23
Averages	1677	85			2616	92	1.58	6.5	29

The complete metabolism data on the hyperthyroid rats, secured after stabilization at the dosage of iodinated casein used, will be found in table 5, which also affords comparisons with the observations secured on the rats prior to dosage. Due to the induced hyperthyroidism, the basal metabolism of the rats increased over a range of from 67 to 116%, averaging 85%. Simultaneously the minimum endogenous urinary nitrogen increased through a range of from 62 to 119%,

TABLE 6

Basal metaoblism of the rats at two different food intake levels

RAT NO.	AT LOWER LEVEL IN PERIOD I (13 GM FOOD/DAY PRIOR TO BASAL)			AT HIGHER LEVEL IN PERIOD III (16 GM FOOD/DAY PRIOR TO BASAL)			INCREASE IN BM AT HIGHER LEVELS OF FOOD INTAKE
	Body weight	Basal heat[1]	R.Q.	Body weight	Basal heat	R.Q.	
	gm	*cal./m²/day*		*gm*	*cal./m²/day*		%
3	266	911	0.70	288	953	0.76	+ 4.6
4	244	842	0.71	256	946	0.72	+ 12.3
6	258	785	0.64[2]	293	786	0.73	+ 0.1
7	250	861	0.69[2]	273	873	0.74	+ 1.4
8	254	867	0.74	263	908	0.70	+ 4.7
9	259	887	0.70	292	927	0.74	+ 4.5
10	261	857	0.72	292	943	0.73	+ 10.0
Average		858			905		+ 5.4

[1] Surface area of the rats was estimated according to the formula of Lee ('29) viz., $S_m{}^2 = 12.54\ W_{gm}{}^{0.60}$.

[2] The heat production in all periods for which R.Q.'s of less than 0.707 were obtained was computed by assuming a heat equivalent of a liter of oxygen of 4.686 cal.

averaging 92%. The ratio of endogenous urinary nitrogen to basal heat did not change significantly, the average of the hyperthyroid state being 1.58 and for the normal state 1.53.

Part of the average increase of 85% in the basal metabolism of the hyperthyroid rats may have been a result of the greater plane of nutrition on which they had been subsisting, since they received 19 gm of food daily as compared to 16 gm in the period of normal treatment. The effect on the basal metabolism of a 3 gm difference in daily food intake is shown in table

6. In all cases the metabolism was higher when the basal condition was established after a period of feeding 16 gm of food daily than after a period of feeding 13 gm. The differences ranged from 0.1 to 12.5% and averaged 5.4%. It may be concluded, therefore, that very little of the elevation in the metabolism of the hyperthyroid rats over the normal rate can be ascribed to the higher plane of pre-feeding.

Table 5 confirms many previous studies in hyperthyroidism by showing a marked creatinuria in the experimental animals. An average of 29% of the total urinary creatinine was present as creatine, a clear indication in itself of an accelerated endogenous nitrogen catabolism. The total creatinine nitrogen averaged 6.5% of the total urinary nitrogen, a value not greatly different from that reported by Smuts ('35) for normal rats, i.e., 7.1%.

The fact that thyroid-active material will increase the minimum endogenous nitrogen metabolism as well as the basal metabolism, and that the increases are approximately in the same proportion, is in harmony with the Terroine-Smuts principle that the two types of maintenance metabolism are related in magnitude, and that the protein requirements for maintenance, as well as the energy requirements, vary with the body surface more nearly than with the body weight, other determining factors remaining the same.

SUMMARY AND CONCLUSIONS

Metabolism experiments were performed on 7 adult male albino rats, first under normal conditions and then under conditions of hyperthyroidism induced by dosage with iodinated casein. The basal metabolism of energy and the output in the urine of nitrogen from endogenous sources only were determined under both normal and hyperthyroid conditions by the usual methods.

Both the basal metabolism and the minimum endogenous nitrogen metabolism are elevated 80 to 90% during hyperthyroidism of the intensity induced. The percentage of total

creatinine of the total endogenous urinary nitrogen is not greatly disturbed, although a marked creatinuria is induced.

The similar effect of thyroid-active material on the basal metabolism and on the minimum endogenous metabolism of nitrogen in the adult animal is in harmony with the Terroine-Smuts principle that the two are related causally and, under normal conditions, vary with body size in a parallel fashion, other determining factors remaining the same.

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ANALYTICAL AND EXPERIMENTAL STUDY OF THE EFFECTS OF INCREASED PROTEIN WITH LIBERAL CALCIUM AND RIBOFLAVIN INTAKES: COMPLETE LIFE CYCLES[1]

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ONE FIGURE

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Slonaker (several researches summarized in '39), in long term experiments with rats on diets containing 10, 14, 18, 22, and 26% protein, found 14% the most advantageous level. While no other work has superseded that of Slonaker, some other findings have raised the question of whether more protein could be utilized to advantage if more attention were given to liberality of intake of mineral elements and vitamins, especially calcium and riboflavin.

Advantage from extra protein thus fed might be detected either by analytical determination of higher levels of protein or riboflavin or both in the body, or by enhanced biochemical functioning as shown in growth, adult vitality, and length of life.

In connection with the use of diets consisting largely or mainly of wheat (or flour) and milk, with the rat as experimental animal, it was found by MacLeod ('26–'27), by Russell ('32), and by one of the present authors (H.L.C., unpublished

[1] Aided by grants from Swift and Company to Columbia University.

[2] Present address: Food and Nutrition Board, National Research Council, Washington, D. C.

data) that the addition of meat to these diets tended to increase growth and reproduction records. Individual differences, however, were such that averages for 15 females on each of two comparable diets proved insufficient for conclusive findings. Later it became possible for us to study the effects of increased protein with much larger numbers of animals, as described below.

Meanwhile, Slonaker ('39) reported upon the effect of different percentages of protein in the diets of 6 generations of rats. He observed (as in his earlier work) better results in rats fed a diet containing 14%, than in those fed 10, 18, 22, or 26% of protein in the air-dry food mixture. Repeatedly, Slonaker cites his finding of 14% as optimum: in one case he adds, "supplemented by a higher percentage of protein for rearing the young," i.e., during lactation. While his extensive observations thus showed a few indications that a protein content slightly higher or lower than 14% might in particular cases have given better results, it seemed evident that for the conditions of his experiments the optimum level of protein intake for lifelong well-being was certainly not over 16%. Experiments on growth alone may, of course, favor much higher levels of intake.

PURPOSE AND PLAN OF THE PRESENT WORK

The purpose of the experiments here reported was to ascertain whether a higher intake of protein than that which appeared optimum in Slonaker's work can be used to increased advantage if the experimental diet also contains liberal proportions of calcium, vitamin A, and riboflavin.

The starting point of these experiments was our laboratory diet 133, consisting of two-thirds ground whole wheat and one-third dried whole milk, with the addition of sodium chloride amounting to 2% of the weight of the wheat and enough calcium carbonate to give the food mixture a calcium content of 0.64%. The riboflavin content was approximately 7.2–7.7μg/gm; the protein, 16%; vitamin A value, 6 I.U./gm. In strict parallel was fed laboratory diet 180, which differed

from diet 133 only in that dry beef muscle protein was added to increase the protein content of the food mixture by one-fourth. This brought the total protein content to 20%, while the riboflavin content was within the above-given range of 7.2–7.7 μg/gm. The beef muscle protein [3] was thoroughly defatted and contained only insignificant amounts of anything other than protein and hygroscopic moisture.

Each of our series of experiments was begun by assembling strictly parallel "families" or "lots" by assignment of litter mates of the same sex and size to each of the two diets above described (diets 133 and 180). The initial age was 28 days. The sexes grew up together, usually three females and two males in a 12 × 15 inch cage, and the age at which each female gave birth to her first young was one of the objective criteria as to whether the difference in diet influenced the rate of development. Weights were recorded weekly. Each female, when she showed pregnancy, was placed in a separate cage and given opportunity to rear as many of her young as she could. As each litter reached the age of 28 days the mother was returned to her permanent cage and the young assigned either for analysis at predetermined ages or to the assembling of new breeding lots to carry the study through a second generation.

As the influence of an experimental diet is sometimes clearer in the second generation, it was desirable that some of these second generation lots be included in the investigation; but not so many as to reduce unduly the number of new samplings drawn from the long-established colony.

RESULTS OF ANALYSES OF EXPERIMENTAL ANIMALS FROM DIFFERENT DIETS AT PREDETERMINED AGES

The averages resulting from the analyses are summarized in tables 1 and 2, the figures in parentheses showing the number of cases entering into each tabulated average. Nitrogen

[3] The beef muscle protein was prepared in the research laboratories of Swift and Company and furnished us through the courtesy of Dr. H. E. Robinson, to whom we are greatly indebted for this cooperation.

(protein), phosphorus, and riboflavin were determined in the muscle and liver of rats from families on each of the diets at the ages of 30, 60, 90, 120, 150, and 180 days.

The muscles of the animals from each diet showed a slight upward trend of nitrogen (protein) and downward trend of riboflavin concentration during the age range covered by these analyses.

At none of the ages here studied did the nitrogen, phosphorus, or riboflavin concentration of muscle or liver appear to be significantly influenced by the difference in the protein content of these diets, although it is known that by means of

TABLE 1

Nitrogen and phosphorus content in per cent of moist muscle and moist liver

AGE	MOIST MUSCLE				MOIST LIVER			
	Nitrogen		Phosphorus		Nitrogen		Phosphorus	
	From diet 133	From diet 180	From diet 133	From diet 180	From diet 133	From diet 180	From diet 133	From diet 180
days	%	%	%	%	%	%	%	%
30	2.92(4)[1]	3.00(4)	0.244(4)	0.238(5)	2.62(4)	2.66(5)	0.269(4)	0.266(4)
60	3.23(3)	3.29(2)	0.229(3)	0.234(2)	3.01(3)	3.04(2)	0.253(2)	0.263(2)
90	3.26(5)	3.14(5)	0.242(5)	0.238(3)	2.98(5)	2.92(5)	0.281(5)	0.290(5)
120	3.34(4)	3.30(2)	0.255(4)	0.246(2)	3.09(4)	3.08(2)	0.296(4)	0.283(2)
150	3.44(3)	3.44(3)	0.235(3)	0.255(3)	3.04(3)	3.16(3)	0.277(3)	0.247(3)
180	3.26(3)	3.36(4)	0.224(3)	0.222(4)	2.75(3)	3.03(4)	0.262(3)	0.226(4)

[1] Numbers in parentheses indicate number of cases.

TABLE 2

Riboflavin in moist muscle and liver

AGE	MOIST MUSCLE		MOIST LIVER	
	From diet 133	From diet 180	From diet 133	From diet 180
days	*µg/gm*	*µg/gm*	*µg/gm*	*µg/gm*
30	4.1(65)[1]	4.2(58)	20.3(64)	19.9(57)
60	3.6(20)	3.4(15)	23.5(20)	25.2(15)
90	3.3(19)	3.4(15)	25.0(20)	23.7(15)
120	3.3(16)	3.1(14)	27.4(16)	25.2(15)
150	2.8(13)	3.1(14)	25.1(14)	22.8(14)
180	2.9(16)	2.9(14)	23.8(19)	22.5(15)

[1] Numbers in parentheses indicate number of cases.

larger differences of protein intake one may influence the concentrations of protein and riboflavin in body tissues (Sarrett, Klein, and Perlzweig, '42; Sherman and Ragan, '48).

The fact that the difference of protein intake here studied did not cause a measurable difference of concentration levels in the body makes especially noteworthy the further fact that this same difference of intake did have measurable effects upon some aspects of biochemical performance as recorded below and in table 3.

While the beef muscle protein kindly furnished us by Dr. Robinson[1] for these experiments was not a chemically pure substance, we see no reason to doubt that its significance here was simply with respect to its protein content. As explained in the above description of the diets, the increase of protein content (diet 180 over diet 133) was effected within a variation in riboflavin or any minor substance of only about one-twentieth of the amount concerned.

In a series of parallel experiments it was found that increasing by one-fourth the phosphorus and riboflavin contents of the diet had no measurable influence upon the growth of the body or upon the nitrogen, phosphorus, or riboflavin content of the tissues. Thus the phosphorus intake was not the limiting factor in the bodily retention of nitrogen, of phosphorus, or of riboflavin, the level of intake of each being presumably within the range of its optimum. Apparently the body's concentration of phosphorus remained somewhat more constant than that of nitrogen (protein) or riboflavin during the normal growth of the rat from the age of 30 to that of 180 days. While this growth undoubtedly involved an increased bodily amount (as distinguished from concentration) of a riboflavin-phosphate-protein compound or compounds, the effects of the increase of dietary protein in these experiments are to be interpreted as essentially protein effects not complicated by any limitation of phosphorus or of riboflavin.

Thus the figures in tables 1 and 2 show that increasing the protein content of the diet from 16% to 20% — an increase

[1] See footnote 3, p. 319.

TABLE 3

Biochemical performance on diets of different protein content

	ON DIET 133 (16% PROTEIN)	C.V.	ON DIET 180 (20% PROTEIN)	C.V.	DIFFERENCE
Gain in weight, 28th–56th days of age					
females (gm)	66.1 ± 0.61 (84)[1]	13	73.9 ± 0.78 (83)	11	7.8 ± 0.99
males (gm)	88.6 ± 1.45 (54)	18	106.2 ± 1.32 (55)	11	17.6 ± 1.96
Age of females at birth of first young (days)	101.9 ± 1.49 (82)	20	98.2 ± 1.21 (80)	17	3.7 ± 1.92
Duration of reproductive life of females (days)	297.0 ± 11.4 (84)	53	366.0 ± 10.5 (84)	40	69.0 ± 15.3
Young borne per female	34.0 ± 1.24 (84)	50	42.8 ± 1.39 (84)	15	8.8 ± 1.86
Young reared per female	23.3 ± 1.01 (84)	60	31.3 ± 1.27 (84)	56	8.0 ± 1.62
Per cent of young reared	68.6 ± 1.73 (82)	34	70.6 ± 1.75 (80)	33	2.0 ± 2.46
Aver. weight of young at 28 days (gm)	46.1 ± 0.09 (1957)	12	50.9 ± 0.05 (2626)	8	4.8 ± 0.10
Length of life					
males (days)	711.0 ± 10.5 (55)	16	728.0 ± 12.7 (55)	19	17.0 ± 16.4
females (days)	783.0 ± 16.2 (83)	28	824.0 ± 11.7 (83)	19	41.0 ± 20.0

[1] Numbers in parentheses indicate number of cases.

of one-fourth over the initial concentration in the food — did not measurably increase the protein, riboflavin, or phosphorus content of the tissues. Yet this difference of protein intake did measurably influence biochemical performance as shown below.

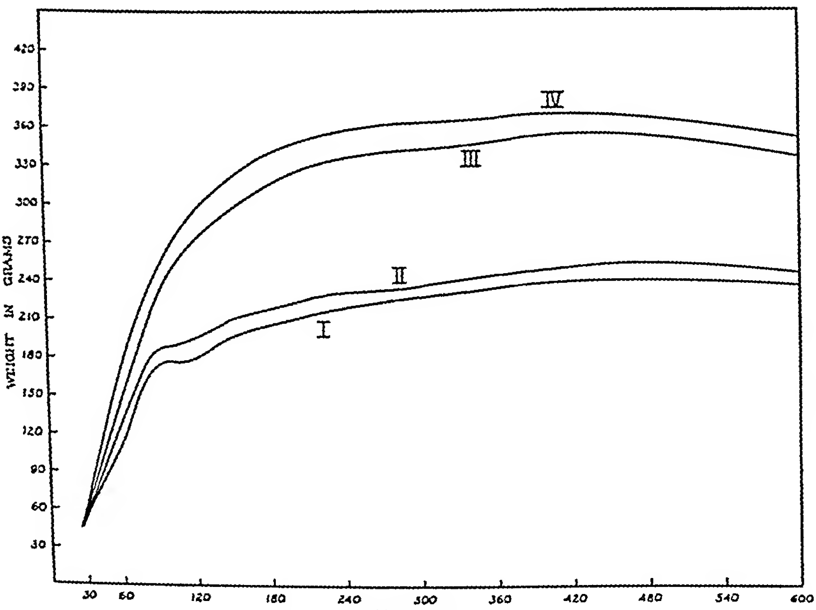

Fig. 1 Body weight curves of the rats, the further reords of which are summarized in table 3: I — females on diet 133 (16% protein); II — females on diet 180 (20% protein); III — males on diet 133; IV — males on diet 180.

INFLUENCE OF THE LEVELS OF PROTEIN INTAKE UPON BIOCHEMICAL PERFORMANCE

The aspects of biochemical performance to which attention has here been especially directed because of their objectivity and measurability are growth, success in reproduction and rearing of the young, and length of life. The initial numbers of experimental animals on each of the two diets were 55 males and 84 females, of which latter no less than 80 (or 95%) of those on each diet reared young. The average data for the animals on each diet with respect to each of the criteria

here measured are summarized in table 3, from which it will be seen that notwithstanding the absence of measurable differences in body composition (tables 1 and 2) certain objective criteria of biochemical performance do show measurable effects of *the difference in the protein content of the diet.* All significant differences here found were in the direction of higher records made by animals from families on diets of higher protein content.

Both sexes unquestionably showed more rapid early growth on the diet with 20% than on that with 16% protein. These differences are for females 8 times and for males 9 times their probable errors. The animals on the higher protein diet also had higher body weights as adults. Weight curves for each sex on each diet are shown in figure 1, the irregular data of old age being omitted to save space.

Correspondingly, females on the diet with the higher percentage of protein bore their first young at an earlier average age. Here, however, the difference is relatively much less, only twice its probable error, so that by itself it would not be judged statistically significant.

Females on the diet of higher (20%) protein content showed longer average duration of reproductive life, and here the difference is sufficiently large (4.5 times its probable error) to be judged statistically significant.

The numbers of young borne and reared were also higher for the females on the diet with 20% than for those on that with 16% of protein, and here the differences (4.7 and 5.0 times their probable errors, respectively) are statistically significant in both cases.

The percentage of young reared was essentially the same for the families on the two diets.

The average weight of the young at the conventional separation age of 28 days is unquestionably greater in the families on the diet with 20% than in those on the ration with 16% of protein, the difference being 48 times its probable error.

On each diet the females lived longer than the males, as is usual in this colony.

Neither with the males nor with the females did the increased protein content of the diet significantly influence the length of life.

DISCUSSION

It is clear that raising the protein content of the diet by one-fourth, from an initial 16% to 20%, by addition of beef muscle protein to the air-dry food mixture increased the rate of growth and (less markedly) the adult size of the experimental animals without measurably increasing the concentration of protein, riboflavin, or phosphorus in the body tissues.

Yet the effect of the extra protein, while not demonstrable by direct analyses of the tissues, did measurably increase not only the growth and size of both sexes, but also the reproduction records of the females.

Here the long-term quantitative observation of the body's biochemical performance proved a more delicate method of finding the effects of the dietary change than did direct chemical analysis of tissue.

The fact that a higher protein intake level was used more advantageously here than in Slonaker's work is probably due to more liberal calcium, riboflavin, and vitamin A values in our basal diet.

How may one picture a chemical improvement not demonstrable by dircet chemical analyses? Three possible concepts suggest themselves, any or all of which may be true.

The concentration principle suggests that increased protein intake may raise the concentration of proteins or of some of their derivatives in the body by a margin too small to be found by present methods of analysis yet which, continuing for a lifetime, may measurably influence the record of biochemical performance.

A second possibility is that the flowing of a larger amount of protein through the metabolic process, even at an unchanged concentration level, may tend to raise the *tone* of the life process, possibly by interaction with other nutrients.

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A second possibility is that the flowing of a larger amount of protein through the metabolic process, even at an unchanged concentration level, may tend to raise the *tone* of the life process, possibly by interaction with other nutrients.

A third hypothesis is that the larger animal which tends to result from the higher protein intake may have some advantage of physical capacity over a smaller individual of the same strain and chemical composition. Comparison of the larger and smaller animals on the same diet indicates that the former show a slight advantage in their breeding records but no significant difference in length of life.

SUMMARY AND CONCLUSIONS

In view of recent advances in the biochemistry of calcium, riboflavin, and vitamin A, long-term experiments have been made (with rats) to test the effect of increasing the protein content of the diet from an initial 16% to 20% in the air-dry food mixture, the basal diet being liberal in its calcum, riboflavin and vitamin A content.

With this basal diet the increase of protein intake proved more advantageous in its long-term effects than had been the case in the corresponding work by Slonaker ('39). Animals receiving food containing 20% as contrasted with 16% of protein showed increased growth and attained a larger adult size and the females made higher reproduction records, though direct chemical analyses of body tissues showed no measurable change of concentration of protein, phosphorus, or riboflavin. Hypotheses relating to the greater precision of some long-term criteria than of direct chemical analysis by present-day methods are discussed briefly.

The moderate difference of protein intake here studied did not significantly influence the length of life or the percentage of young reared, though the higher protein diet did result in somewhat larger size in both sexes and in the rearing of more and larger young.

ACKNOWLEDGMENT

The executive guidance of Professor A. W. Thomas, and the efficient services of all who took part in the work, whether as research assistants or volunteers, are gratefully acknowledged.

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NINE ESSENTIAL AMINO ACIDS IN PURE VARIETIES OF WHEAT, BARLEY AND OATS[1]

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Available data (Baumgarten, Mather and Stone, '46; Block and Mitchell, '46; Schweigert, '47, '48) on the essential amino acid content of cereal grains are based largely on analyses done on limited numbers of samples of indefinite origin. Assay results for large numbers of samples of known origin and variety are required to provide information regarding variability in quality of grain protein and possible relationships between such factors as soil, climate, variety and species and the essential amino acid composition of cereal grain proteins. Results of microbiological assays conducted on 9 samples of wheat, barley and oats of known variety and origin are reported in the present paper. Assays were repeated a sufficient number of times to permit statistical analysis of the results.

EXPERIMENTAL

Assay material

The grains studied were Marquis wheat, Newal barley and Victory oats grown in 1945. Samples were selected to cover a wide range in protein content and to represent grains grown

[1] Part of the data in this paper was taken from a thesis submitted by William Lobay in partial fulfillment of the requirements for the M. Sc. degree. Financial assistance was received from the Committee on Agricultural Research Grants, University of Alberta, and from the National Research Council of Canada. Paper 263 of the Associate Committee on Grain Research.

under a variety of soil and climatic conditions. With a few exceptions they were obtained from University of Alberta test plots and from Dominion Illustration and Experimental Stations located in representative soil zones throughout the province of Alberta.

Assay methods

Nitrogen and dry matter. Nitrogen determinations were done on one gram samples by the Kjeldahl-Gunning-Arnold method (A.O.A.C., '45) using mercuric oxide as catalyst. Two or more determinations in duplicate were made on each sample. Dry matter was determined by heating finely ground samples in a gravity convection oven for 18 hours at 105°C.

Hydrolysates. Hydrolysates were prepared by heating one gram samples of finely ground grain in 25 ml of 2N hydrochloric acid in an autoclave for 10 hours at 15 pounds pressure. The results of preliminary tests on duration of hydrolysis showed that the level of α-amino nitrogen, as measured by the Van Slyke nitrous acid method, reached a maximum after 7 hours in the autoclave, and remained constant at intervals between 7 and 20 hours.

Amino acids. A minimum of two sets of paired hydrolysates of each sample were assayed microbiologically for 9 essential amino acids, using *Lactobacillus arabinosus* 17–5 for leucine, isoleucine, valine and phenylalanine; *Streptococcus faecalis* R for methionine, threonine, histidine and arginine; and *Leuconostoc mesenteroides* P-60 for lysine. The media used and the assay technique followed were the same as those employed by Riesen, Clandinin, Elvehjem and Cravens ('47).

RESULTS AND DISCUSSION

The results of assays done on 9 samples of Marquis wheat, Newal barley and Victory oats, expressed in terms of per cent of the total nitrogen of a sample contributed by the nitrogen of each of 9 essential amino acids, are summarized in table 1. Values listed for each sample and each amino acid represent the average of from three to 6 assays completed in duplicate.

Statistical tests for significance (Goulden, '39; Snedecor, '46) were applied to the original data when the mean values listed in table 1 suggested the possibility of differences between species or between samples of one variety of one species.

Sample differences within a variety of one species

Inspection of the data in table 1 shows that in all three grains some variation may exist in the per cent of total nitrogen contributed by the nitrogen of a given amino acid in different samples of one variety. This variability appears to be most marked for the amino acids lysine, arginine, valine and phenylalanine. F values, derived for variance due to samples within a variety of one species, were significant beyond the 1% point for lysine in all three grains, for arginine and valine in Marquis wheat and Newal barley, and for phenylalanine in Victory oats and Newal barley.

Samples in table 1 are listed in order of nitrogen content and it may be noted that in the wheat and barley samples there is a trend towards a decrease in per cent lysine nitrogen of total nitrogen with increasing levels of the latter. The values reported by Schweigert ('48) for lysine in 5 samples of wheat of different protein content also suggest the possibility of an inverse relationship between total protein and per cent lysine of protein. Regression coefficients were calculated for the present data and it was found that in the wheat samples lysine nitrogen as per cent of total nitrogen decreased 0.021% for each increase of 1% in total nitrogen, but neither this coefficient nor that of —0.019 derived for barley was found to be significant at the 5% point by the t test.

It is recognized (Anderson, '39–'41) that the total protein content of grains is affected by the conditions of soil and climate under which they are grown; for example, it is evident from table 1 that the samples grown in the gray-wooded soil zone contained less protein than those grown in the brown soil zones. The present data do not, however, demonstrate the existence of a significant correlation between soil zone and

TABLE 1

Per cent of total nitrogen contributed by 9 essential amino acids in grains of different protein content.
(Data are reported on a moisture-free basis)

SAMPLE NO.	SOIL ZONE [1]	TOTAL N	AMINO ACID NITROGEN FRACTION OF TOTAL NITROGEN									
			Leucine	Isoleucine	Valine	Phenylalanine	Methionine	Threonine	Histidine	Arginine	Lysine	Total
		%	%	%	%	%	%	%	%	%	%	%
Marquis wheat												
W- 3	G.	1.94	4.23	3.14	3.56	2.68	0.72	2.01	2.89	9.38	2.89	31.50
W- 4	D.B.	2.64	4.39	3.18	3.49	2.61	0.76	2.08	2.65	9.47	2.96	31.59
W- 2	Bl.	2.85	4.14	3.09	3.30	2.70	0.67	2.00	2.63	9.12	2.67	30.32
W-17	D.B.	3.00	4.20	2.97	3.20	2.67	0.67	1.90	2.60	9.07	2.70	29.98
W- 6	D.B.	3.09	4.34	3.01	3.20	2.62	0.71	1.94	2.62	8.84	2.56	29.84
W- 9	D.B.	3.12	4.39	3.05	3.33	2.69	0.67	1.96	2.66	8.97	2.60	30.32
W-15	D.B.	3.19	4.01	2.88	3.20	2.67	0.69	1.88	2.57	8.81	2.57	29.28
W-14	D.B.	3.35	4.24	2.96	3.22	2.54	0.66	1.97	2.66	8.72	2.57	29.54
W- 1	L.B.	4.03	4.22	2.80	3.20	2.71	0.67	1.89	2.58	8.51	2.61	29.19
Mean		3.02	4.24	3.01	3.30	2.65	0.69	1.96	2.65	8.99	2.68	30.17
Newal barley												
B-10	G.	1.83	4.21	2.73	3.55	2.24	0.71	2.19	2.30	10.05	4.21	32.19
B-19	G.	2.00	4.50	2.85	3.65	2.55	0.75	2.35	2.35	9.70	3.90	32.60
B-21	G.T.	2.07	4.54	2.80	3.77	2.56	0.77	2.32	2.42	10.24	4.01	33.43
B-25	G.T.	2.32	4.48	2.76	3.62	2.54	0.73	2.37	2.50	9.44	3.84	32.28
B-11	Bl.T.	2.56	4.26	2.70	3.56	2.19	0.70	2.23	2.38	9.57	4.06	31.65
B- 9	D.B.	2.58	4.38	2.71	3.57	2.60	0.70	2.25	2.44	9.54	3.64	31.83
B-14	D.B.	2.81	4.48	2.71	3.59	2.71	0.71	2.21	2.53	9.04	3.63	31.61
B-20	Bl.	2.98	4.46	2.72	3.59	2.72	0.74	2.22	2.35	8.76	3.66	31.22
B- 1	L.B.	3.10	4.23	2.71	3.58	2.84	0.61	2.07	2.36	9.03	3.61	31.04
Mean		2.47	4.39	2.74	3.61	2.55	0.71	2.25	2.40	9.49	3.84	31.98
Victory oats												
O-10	G.	1.51	4.37	3.31	4.11	2.32	0.66	2.12	2.05	11.85	3.78	34.57
O-19	G.	1.67	4.43	3.29	4.19	2.34	0.72	2.16	2.16	11.80	3.77	34.86
O-24	G.	1.99	4.72	3.37	4.27	2.56	0.75	2.26	2.21	12.01	3.82	35.97
O-12	G.	2.16	4.68	3.43	4.21	2.50	0.69	2.22	2.32	12.59	3.98	36.62
O-26	G.T.	2.31	4.55	3.25	4.11	2.34	0.69	2.21	2.25	11.95	3.90	35.25
O- 7	D.B.	2.50	4.72	3.28	4.20	2.56	0.72	2.24	2.40	12.52	3.76	36.40
O- 8	D.B.	2.54	4.88	3.31	4.29	2.52	0.71	2.28	2.40	12.68	4.02	37.09
O-16	D.B.	2.71	4.95	3.36	4.50	2.77	0.66	2.21	2.29	12.40	4.10	37.24
O-13	L.B.	3.15	4.67	3.18	4.00	2.44	0.67	2.19	2.44	12.67	3.75	36.01
Mean		2.28	4.66	3.31	4.21	2.48	0.70	2.21	2.28	12.27	3.88	36.00

[1] G. = gray-wooded; G.T. = gray transitional; Bl = black; Bl.T. = black transitional; D.B. = dark brown; L.B. = light brown.

grain protein quality as measured by the proportion of the 9 essential amino acids under consideration. In a recent paper Sheldon, Blue and Albrecht ('48) indicate that the quality of lespedeza and alfalfa protein is affected by soil zone and by fertilizer treatment, but inasmuch as the data presented do not show the total nitrogen or protein content of the material considered, it is not clear whether their experiments demonstrate marked changes in quality, or simply in quantity, of protein produced on different soils and under different treatments.

TABLE 2

Amino acid content of the protein (N × 6.25) of 9 samples of Marquis wheat, Newal barley and Victory oats. (Data are reported on a moisture-free basis)

	MARQUIS WHEAT		NEWAL BARLEY		VICTORY OATS	
	Mean	Range	Mean	Range	Mean	Range
	%	%	%	%	%	%
Leucine	6.4	6.0–6.6	6.6	6.2–6.8	7.0	6.6–7.3
Isoleucine	4.5	4.2–4.8	4.1	4.0–4.2	5.0	4.8–5.2
Valine	4.4	4.3–4.8	4.9	4.7–5.0	5.7	5.4–5.9
Phenylalanine	5.0	4.8–5.1	4.8	4.2–5.2	4.7	4.3–5.1
Methionine	1.2	1.1–1.3	1.2	1.0–1.3	1.2	1.1–1.3
Threonine	2.7	2.6–2.8	3.0	2.8–3.2	3.0	2.9–3.1
Histidine	1.6	1.5–1.7	1.4	1.4–1.5	1.3	1.2–1.5
Arginine	4.5	4.2–4.7	4.7	4.4–5.1	6.1	5.8–6.3
Lysine	2.2	2.1–2.5	3.2	3.0–3.5	3.3	3.1–3.4
Total 9 amino acids	32.5		33.9		37.3	

Species differences

In table 2 the assay results are briefly summarized in terms of per cent amino acid of total N × 6.25. From these data or those in table 1 it may be seen that in the samples under assay differences exist among the proteins of Marquis wheat, Newal barley and Victory oats with respect to concentrations of certain of the amino acids. For example, the mean lysine value determined for Newal barley and Victory oat protein is 1.4 to 1.5 times that obtained for the protein (N × 6.25) of Marquis wheat.

Fiducial limits were calculated from the assay data—a separate standard error being calculated for each class of data—to determine the probability of the existence of differences among the proteins of the three grains with respect to concentrations of lysine, arginine, valine, threonine, leucine, isoleucine and histidine. For the last three amino acids the probability of inter-species differences was less than 20:1. The comparisons which showed a probability greater than 20:1, i.e., where there was no overlapping of the respective 5% fiducial limits of the two means being compared, are presented in table 3.

TABLE 3

Probability of inter-species differences

Concentration of	PROBABILITY THAT		ODDS IN FAVOR
	In	Exceeds that in protein of	
Lysine	Victory oats	Marquis wheat	> 100:1
Lysine	Newal barley	Marquis wheat	> 20:1
Arginine	Victory oats	Marquis wheat	> 20:1
Arginine	Victory oats	Newal barley	> 20:1
Valine	Victory oats	Marquis wheat	> 20:1
Valine	Newal barley	Marquis wheat	> 20:1
Valine	Victory oats	Newal barley	> 20:1
Threonine	Victory oats	Marquis wheat	> 20:1
Threonine	Newal barley	Marquis wheat	> 20:1

These results, based on assays of 9 samples of each grain, indicate that the protein of Marquis wheat is inferior to that of either Victory oats or Newal barley with respect to content of lysine, and that it is probably inferior with respect to two other amino acids, threonine and valine, which may be required in supplemental amounts in grain rations. Although the significance of this relationship may be small in practical feeding, since all three grains are relatively poor sources of these amino acids, the possibility is nonetheless indicated that the use of protein supplements of high quality may be especially important in livestock and poultry rations containing a high proportion of wheat. In this connection it should,

however, be noted that Doty, Bergdoll, Nash and Brunson ('46) have shown that the amounts of certain amino acids in corn protein are, to some extent, affected by the genetic constitution of the plant. If future work with other cereal grains should demonstrate the existence of significant varietal differences within species, another complicating factor would have to be taken into consideration in any attempt to draw conclusions regarding the relative adequacy of the protein of different species of céreal grain.

SUMMARY

1. Results are reported of microbiological assays for 9 essential amino acids in 9 samples of one variety each of wheat, barley and oats.

2. Variability was observed in the proportion of the total nitrogen contributed by the nitrogen of a given amino acid in different samples of a variety within a species. This variability was most evident for the amino acids lysine, arginine, valine and phenylalanine.

3. The possibility is discussed that the fraction of the total nitrogen contributed by the nitrogen of lysine may decrease as the total nitrogen content of grain increases.

4. Assay values obtained with samples grown under a wide range of soil and climatic conditions failed to demonstrate the existence of a significant correlation between soil zone and grain protein quality as measured by the proportion of 9 essential amino acids in grain proteins.

5. Evidence is presented indicating that the protein of Marquis wheat may contain significantly less of certain of the essential amino acids, notably lysine, than do the proteins of Victory oats or Newal barley.

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STUDIES ON THE ADEQUACY OF HUMAN MILK AND ARTIFICIAL HUMAN MILK FOR THE RAT[1]

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ONE FIGURE

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The results of studies of the composition of human milk have shown that wide differences exist between the nutritive properties of human milk and those of cow's milk. In spite of these differences, the trend in recent years has been toward the use of cow's milk as the major ingredient in formulas for the feeding of very young infants.

It appeared desirable, therefore, to conduct further experiments on the adequacy of infant feeding formulas composed of ingredients derived largely from cow's milk. Since the opportunities to conduct such experiments on the infants themselves are obviously limited, much of the preliminary work on such investigations must, of course, be carried out with laboratory animals.

Since few studies of this character have been conducted with artificial human milks, little evidence is available as to the applicability of results obtained with an experimental animal, such as the rat, to the actual needs of an infant.

The studies presented in this report were conducted to compare, by rat feeding experiments, the nutritive properties

[1] This work was aided by a grant to Cornell University from the Western Condensing Company, San Francisco. The work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry, Cornell University.

of dehydrated human milk with those of artificial milk mixtures designed to simulate human milk. The purpose of such comparisons was not only to evaluate the respective nutritive properties of the diets for the rat, but also to provide information concerning the suitability of the rat for evaluating materials intended for infant feeding.

EXPERIMENTAL PROCEDURE

Protein analyses conducted on several samples of human milk verified the findings of others that considerable variability is encountered in protein content. Human colostrum (milk produced during the first 10 days after parturition) contains considerably more protein than mature milk (milk produced after the 10th day).

An artificial human milk was compounded in dried form by the addition of lactalbumin, lactose, and unsalted butter to dried whole cow's milk, to give a gross chemical analysis similar to that of the average human colostrum. Thus, this artificial human milk contained 13.4% protein, 28.7% fat, and 51.5% lactose. The ratio of casein to lactalbumin was calculated to be 5:7, which is the ratio present in human milk. Minerals and known vitamins were added to this artificial milk in amounts adequate to meet all known requirements of the rat.

Experiment 1

Previous work (Mitchell and Dodge, '35; Ershoff and Deuel, '44; and others) has shown that the rat cannot tolerate high levels of lactose such as are characteristic of human milk. Also, it is generally accepted that in order to meet the amino acid requirements of the rat, the diet should contain the equivalent of 18% of casein. Therefore, the first experiment was conducted to determine if an increased protein level (18%) or additional methionine (0.44% DL-methionine) would increase rat growth when these supplements were added (1) to the basal artificial human milk and (2) to similar diets in which

part of the lactose had been replaced by glucose. The composition of the diets is presented in table 1.

The experiment was conducted with 4-week-old white rats (Sprague-Dawley) of mixed sex, three male and three fe-

TABLE 1

Composition of diets for experiment 1

LOT NO.	COMPONENTS[1]		PROTEIN	FAT	LACTOSE	GLUCOSE
		%	%	%	%	%
1	Dried whole cow's milk	100	26.4	28.69	36.60	
2	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Lactalbumin	8.9	6.8	0.48	0.15	
	Lactose	42.26			42.26	
	Butter	21.04		21.04		
	Totals		13.4	28.69	51.56	
3	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Casein	2.1	1.85			
	Lactalbumin	12.6	9.55	0.67	0.21	
	Lactose	36.65			36.65	
	Butter	20.85		20.85		
	Totals		18.00	28.69	46.01	
4	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Lactalbumin	8.9	6.8	0.48	0.15	
	Lactose	41.82			41.82	
	Butter	21.04		21.04		
	DL-methionine	0.44	0.44			
	Totals		13.84	28.69	51.12	
5	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Lactalbumin	8.9	6.8	0.48	0.15	
	Glucose	42.26				42.26
	Butter	21.04		21.04		
	Totals		13.4	28.69	9.30	42.26
6	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Casein	2.1	1.85			
	Lactalbumin	12.6	9.55	0.67	0.21	
	Glucose	36.65				36.65
	Butter	20.85		20.85		
	Totals		18.00	28.69	9.36	36.65
7	Dried whole cow's milk	25.0	6.6	7.17	9.15	
	Lactalbumin	8.9	6.8	0.48	0.15	
	Glucose	41.82				41.82
	Butter	21.04		21.04		
	DL methionine	0.44	0.44			
	Totals		13.84	28.69	9.30	41.82

[1] The following supplements were added to all experimental diets: Na_2HPO_4, 1.10%; $CaCO_3$, 0.70%; plus 1% of the following mixture: Starch, 6.9 gm; $FeSO_4 \cdot 7H_2O$, 1.2 gm; $MnSO_4 \cdot 4H_2O$, 1.44 gm; $CuSO_4 \cdot 5H_2O$, 0.078 gm; choline chloride, 1.0 gm; Ca pantothenate, 0.005 gm. Thiamine chloride in the amount of 0.001 gm was added to all diets.

male rats per lot. The rats were housed in wire cages in a thermostatically controlled room. Feed and water were supplied ad libitum. The experiment was conducted for a 4-week period. The rats were weighed weekly, and daily feed consumption was recorded. The results of the experiment are presented in table 2.

TABLE 2

Comparison of artificial human milk with whole cow's milk for rat growth (experiment 1)

LOT OR DIET NO	TREATMENT		INITIAL WT. (4 weeks)	FINAL WT. (8 weeks)	GAIN IN WT. FOR THE 4-WK. PERIOD	EFFICIENCY OF FOOD UTILIZATION
			gm	*gm*	*gm*	*gm food/ gm gain*
1	100%	whole cow's milk	70.16	182.83	112.67	2.32
	Artificial human milk containing:					
2	13.4%	Protein Lactose	69.33	117.49	48.16	4.32
	Modified artificial human milks containing:					
3	18.0%	Protein Lactose	71.66	116.83	45.17	4.49
4	13.4%	Protein + Methionine Lactose	68.2	123.4	55.2	4.35
5	13.4%	Protein Glucose	75.5	153.0	77.5	3.72
6	18.0%	Protein Glucose	70.0	156.8	86.8	3.53
7	13.4%	Protein + Methionine Glucose	76.2	155.5	79.3	3.64

The data show that none of the experimental diets was entirely adequate for the rats, since the positive control lot receiving whole cow's milk enriched with certain vitamins and minerals produced greater gains than did any of the other lots.

Since the whole cow's milk diet contained more lactose than diets 5, 6 and 7, the lower growth in the latter three lots of rats could not be explained on the basis of excess lactose. The whole milk diet contained 26.4% protein, while the highest protein level among the experimental lots was 18%. This might be interpreted to indicate that the former protein level was more nearly optimum for the rat than was the latter. However, 18% protein has long been accepted as being adequate for the rat. On the other hand, the whole milk diet contained 4 times as much of the unidentified vitamins present

TABLE 3

Effect upon rat growth of yeast and liver paste supplementation of modified artificial human milk (experiment 2)

LOT NO.	NO. RATS PER LOT	TREATMENT	INITIAL WT. (4 weeks)	FINAL WT. (8 weeks)	GAIN IN WT.
			gm	*gm*	*gm*
1	5	None [1]	63.6	128	64.4
2	4	2% dried brewers' yeast	58.5	142	83.5
3	4	0.5% liver paste	60.0	139	79.0
4	5	Yeast + liver paste	59.5	150.3	90.8
5	3	Whole cow's milk	65.7	149	83.3

[1] Basal diet was diet no. 6 from experiment 1, an artificial human milk modified to contain 18% protein and 36.65% glucose.

in milk as did any of the experimental diets, since the only source of these factors in the experimental diets was from the 25% whole milk used.

Accordingly, a second experiment was conducted to determine whether additional unidentified vitamins are required for optimum rat growth on a diet such as that used for lot no. 6 (see table 1).

Experiment 2

This experiment was conducted entirely with female rats, three to 5 rats per lot. Diet no. 6 from experiment 1 was fed alone and supplemented with 2% dried brewers' yeast and

TABLE 1

Rat growth on artificial human milks plus yeast as compared to that on whole cow's milk (experiment 3)

LOT NO.	TREATMENT[1]		INITIAL WT. (3 weeks)	FINAL WT. (7 weeks)	GAIN IN WT. FOR THE 4-WK. PERIOD	EFFICIENCY OF FOOD UTILIZATION
			gm	*gm*	*gm*	*gm food/ gm gain*
1	100%	whole cow's milk	42.3	147.7	105.4	2.63
	Artificial human milk containing:					
2	13.4%	Protein Lactose	42.2	102.3	60.1	4.48
	Modified artificial human milks containing:					
3	18.0%	Protein Lactose	42.0	107.7	65.7	4.39
4	13.4%	Protein + Methionine Lactose	43.0	99.3	56.3	5.14
5	13.4%	Protein Glucose	42.8	128.8	86.0	3.30
6	18.0%	Protein Glucose	42.8	144.4	101.6	2.96
7	13.4%	Protein + Methionine Glucose	42.7	145.7	103.0	2.90

[1] Diets the same as those used in experiment 1 with the following supplements added to all experimental diets: Na_2HPO_4, 1.10%; $CaCO_3$, 0.70%; dried brewers' yeast, 2.0%; plus 1% of the following mixture: starch 6.9 gm; $FeSO_4 \cdot 7H_2O$, 1.2 gm; $MnSO_4 \cdot 4H_2O$, 1.44 gm; $CuSO_4 \cdot 5H_2O$, 0.078 gm; choline chloride, 1.0 gm; Ca pantothenate, 0.005 gm; thiamine chloride, 0.001 gm; folic acid, 0.0002 gm. Biotin in the amount of 0.0002 gm was added to all diets.

with 0.5% liver paste[2] alone and in combination. Dried whole cow's milk enriched with vitamins and minerals was again fed as the positive control. The experimental outline and results are presented in table 3. These results show that some nutrient present in both dried brewers' yeast and in the liver

[2] A 95% alcohol soluble liver fraction prepared by Wilson and Company, Chicago, Illinois.

paste is necessary for optimum growth in rats fed this type of diet.

Experiment 3

In view of the discovery that the artificial human milk was deficient in an unidentified factor or factors present in dried brewers' yeast and liver paste, a third experiment was conducted using the same diets and experimental plan used in experiment 1 except that 2% of dried brewers' yeast was added to all diets. This experiment was conducted with weanling white rats of mixed sex, three male and three female rats per lot. The results of the experiment are presented in table 4.

It is apparent from these results that the addition of dried brewers' yeast to the 18% protein-glucose diet (lot 6) produced a growth rate almost equivalent to that obtained with whole cow's milk. In fact, an equivalent rate of gain was obtained with the 13.4% protein-glucose lot (lot 7) when sufficient methionine was added to meet the requirements of the rat for this amino acid. On the other hand, all lots (2, 3 and 4) receiving the high level of lactose did not make normal gains even in the presence of dried brewers' yeast. This indicates that, at least during the first month after weaning, the rat cannot utilize lactose sufficiently well to maintain a normal growth rate on lactose levels much above 40%, even though the diet is adequate in all other nutrients. This fact should be kept in mind in evaluating all other experiments presented in this report.

Experiment 4

This experiment was conducted to compare the growth-promoting effects of dried mature human milk[3] with that of dried whole cow's milk. Both milks were supplemented with the vitamins and minerals added to the cow's milk in the previous experiment. Another lot received the human milk supple-

[3] Obtained from the Dispensary for Mothers' Milk, Louisville, Kentucky. The milk was shipped by air freight in the frozen state and was dried under vacuum at temperatures well below freezing.

mented with 2% yeast and 0.5% liver paste. The experiment was conducted with weanling white rats (Sprague-Dawley), three male and three female rats per lot in the group receiving cow's milk and two rats of each sex in the lots receiving human milk. The experimental conditions were the same as those used in the earlier experiments. The diets used and the experimental results are described in table 5.

The results of the experiment show that the rats receiving mature human milk failed to grow at all. The addition of 2%

TABLE 5

Growth failure of rats on mature human milk alone and supplemented with yeast and liver paste (experiment 4)

TREATMENT [1]	BODY WEIGHT		WEIGHT GAIN IN 4 WKS.	FEED CONSUMED PER RAT. IN 4 WKS.
	Average initial	Average at 4 wks.		
	gm	gm	gm	gm
Cow's milk	46.0	168.7	122.7	263
Mature human milk	45.5	42.0	— 3.5	110
Mature human milk + 2% yeast + 0.5% liver paste	44.8	43.5	— 1.3	126

[1] Supplemental vitamins and minerals as described in footnote to table 4, omitting yeast, Na_2HPO_4, and $CaCO_3$.

yeast and 0.5% liver paste to the mature human milk failed to improve it as a diet for the rats. Growth failure of the rats on the human milk diets could not have been due to inanition, since the rats on these diets ate almost as much feed in proportion to their size as the rats on the whole cow's milk diet. The feed consumption in all lots was consistent throughout the experiment. There was no evidence of loss of appetite at any time.

Experiment 5

Since the casein of cow's milk is a much better source of factor S, a growth factor for chicks, than is yeast or liver paste

(Scott, Norris and Heuser, '47), it was decided to conduct an experiment to determine if factor S is required by the rat and is deficient in mature human milk. Accordingly, one lot of rats received human milk alone. Another received human milk plus 5% crude casein. A third lot received human milk plus the pure amino acids equivalent to 5% casein. Each lot contained two of the rats which had been on the human milk diets in experiment 4.

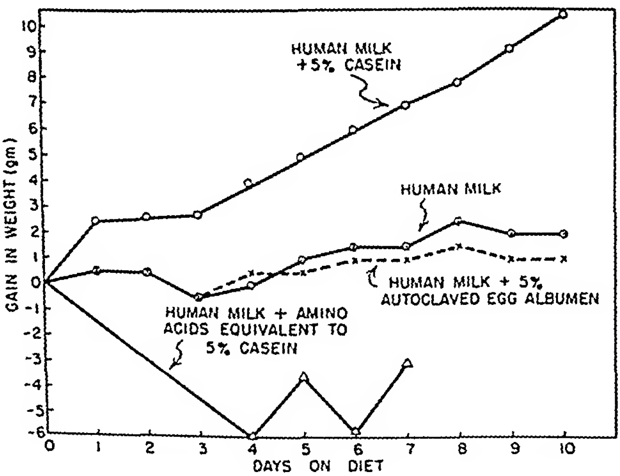

Fig. 1 Results of experiment 5 showing growth obtained when mature human milk was supplemented with crude casein, a source of factor S.

Since the human milk used in these experiments contained only 9% protein, it was considered possible that a growth response due entirely to the additional protein provided might be obtained from supplementation with 5% casein. Therefore, as a second check on this, one lot was fed human milk plus 5% autoclaved egg albumen, a relatively poor source of factor S. In this way the protein was increased to approximately the same level as prevailed for the lot receiving casein without the addition of factor S.

The results of this experiment, presented in figure 1, show that neither egg albumen nor the amino acids caused any growth, but that supplementation with casein produced a good

rate of growth. These results indicate, therefore, that the rat does require factor S and that mature human milk is deficient in this factor.

Experiment 6

This experiment was conducted to determine if human colostrum promotes growth in rats without supplementation with factor S. The human milk for this study[4] was collected largely from mothers who were in their first week to 10 days of lactation. This milk differed from that used in experiments 4 and 5, which was largely mature milk. The protein content of the dried colostrum was found to be 11.9%, approximately 3% higher than that in the human milk used in the earlier experiments.

TABLE 6

Results of experiment 6 showing slow growth of rats on human colostrum not improved by additional casein (factor S)

LOT NO.	TREATMENT[1]	INITIAL WT. (3 weeks)	FINAL WT. (6 weeks)	GAIN IN WT.
		gm	*gm*	*gm*
1	Human milk (colostrum)	50.2	78.5	28.3
2	Human colostrum + 5% autoclaved egg albumen	51.0	71.0	20.0
3	Human colostrum + 5% casein	51.5	76.0	24.5
4	Human colostrum + amino acids equivalent to 5% casein	51.7	74.8	23.1
5	*Artificial human milk* (12% protein)	51.3	72.0	20.7
6	Artificial human milk (13.4% protein)	53.5	97.5	44.0
7	Whole cow's milk	50.1	125.5	75.4

[1] Supplemental vitamins and minerals as in experiment 4, table 5.

Two male and two female weanling white rats were used in each lot in the experiment. One lot received the human milk

[4] Also obtained from the Dispensary for Mothers' Milk, Louisville, Kentucky.

alone. Three other lots received human milk supplemented with 5% egg albumen, 5% casein, and amino acids equivalent to 5% casein, respectively. A fifth lot received the artificial human milk in which the protein level was adjusted to 12% by omission of part of the lactalbumin. This was done to make the artificial human milk have a protein content comparable to that of human colostrum. The sixth lot received the artificial human milk containing 13.4% protein, and the seventh lot received whole cow's milk as a positive control. The results of the experiment are presented in table 6.

These results show that human colostrum promotes growth in rats which is as good as that obtained with an artificial human milk having the same protein content as human colostrum, though not as good as that obtained on the artificial human milk containing 13.4% protein. The rat growth on human colostrum was not affected by addition of casein, indicating that this material is not deficient in factor S for the rat.

DISCUSSION

Experimental results have been presented which show that mature human milk, even when supplementd with vitamins and minerals to meet the known requirements of the rat for these nutrients, is entirely unsuitable as a diet for the rat. On the other hand, human colostrum, supplemented in like manner, does support a slow rate of growth. An artificial human milk formulated to contain the same gross chemical composition as that of human colostrum promoted approximately the same growth rate. Artificial human milk containing a slightly higher level of protein promoted considerably better growth than was obtained on human colostrum; increasing the protein content of mature human milk to approximately the same level did not result in increased growth unless a source of factor S was added also.

Evidence has been presented that the chief deficiencies for the rat in the artificial human milk containing 13.4% protein are methionine and an unknown factor present in dried brew-

ers' yeast. However, even when the diet is supplemented with these substances normal rat growth is not obtained unless a part of the lactose is replaced by glucose.

The failure of rats to grow on mature human milk is probably due to: (1) insufficient protein, more particularly protein containing adequate methionine; (2) a deficiency of the unidentified factor S; and (3) a level of lactose in excess of that which can be tolerated by the rat.

Human colostrum is probably deficient in protein, more especially the amino acid methionine, and contains too much lactose for the rat, but does not appear to be deficient in factor S.

While the rat is obviously a poor experimental animal for use in attempting to evaluate a food for young infants, the results of the studies presented in this report show that, at least as far as the rat is concerned, the artificial human milk formulated to contain a gross composition similar to that of average human colostrum is as nutritious, if not more so, than the human colostrum used in these studies.

All of the animals receiving the high lactose experimental diets developed diarrhea during the first week, which usually disappeared after about 10 days. Many of these experimental animals also developed a peculiar syndrome marked by ruffled fur, alopecia, and a reddened, swollen condition about the eyes resembling the "spectacle eye" reported to be due to biotin deficiency. This was not observed in the rats receiving mature human milk, but appeared in about one-quarter of the rats receiving human colostrum and in about one-half of the rats receiving the artificial human milks. Increased supplementation of these diets with biotin and folic acid had no effect, but supplementation with yeast and liver paste reduced the incidence about 50%.

A condition resembling this following lactose feeding has been described previously by several different groups of research workers (Mitchell, '35, '36; Yudkin and Arnold, '35; Day, '36; and Ershoff and Deuel, '44), except that in the studies described by these investigators the rats also devel-

oped cataracts, while ours did not. Mitchell ('36) and Ershoff and Deuel ('44) reported a strain difference in the development of cataracts and alopecia, both in relation to time of onset and to degree of incidence. Ershoff and Deuel report that on the same purified rations containing lactose as the carbohydrate source, the U.S.C. strain of albino rats survived for a longer period of time with less severe alopecia and cataracts than did the Long-Evans strain. These workers report that young rats fed a diet in which lactose is the sole carbohydrate fail to grow properly and die in from three to 20 days. The work of Mitchell ('35b) and Handler ('47) indicates that the galactose moiety is responsible for the apparent toxic effect of lactose and produces symptoms which are approximately the same as those observed when lactose is fed. Handler stated that increasing the protein or fat content of the diet, or both, increased the rats' survival time and at sufficiently high levels afforded almost complete protection against toxicity. These workers agree that the length of survival and severity of the syndrome are correlated with the severity of diarrhea.

Using an artificial human milk similar in gross composition to those used in the studies presented in this report, Daniel and Harvey ('47) found that rats (Sprague-Dawley) grew at a fair rate, remained normal and healthy in appearance over the experimental period of 6 weeks, and showed evidence of diarrhea only during the first week. They found, on the other hand, that when the mineral content of the diet was increased the animals had diarrhea throughout the whole experimental period. Since these workers reported that, in spite of the diarrhea, their animals appeared normal and healthy, evidence is provided that the alopecia and other symptoms observed by other workers are not necessarily caused by the same condition that produces the diarrhea.

Since the artificial human milk used by Daniel and Harvey contained some dried whole cow's milk, as did ours, and since they supplied the lactose and lactalbumin by means of a nondialyzable fraction from whey, it is possible that their diet

contained an unidentified factor required by the rat on a lactose diet which was missing from the purified diets used by Ershoff and Deuel ('44) and others.

The growth results obtained by Daniel and Harvey were similar to those obtained by us when dried brewers' yeast was added to our artificial human milk basal diet, indicating that the non-dialyzable factor in whey may be the same as that present in brewers' yeast.

The presence of such a factor, together with the high fat level, in the diets used by Daniel and Harvey and by us may explain the lack of mortality observed for animals on these diets.

Further evidence in support of this is the fact that the rats receiving mature human milk (one of the diets having the highest lactose content) did not exhibit any deficiency symptoms other than failure to grow.

SUMMARY

Experimental results have been presented which show that mature human milk, even when supplemented with vitamins and minerals to meet the known requirements of the rat for these nutrients, is entirely unsuitable as a diet for the rat. On the other hand, human colostrum, supplemented in like manner, does support a slow rate of growth. An artificial human milk formulated to contain the same gross chemical composition as that of human colostrum promoted approximately the same growth rate. An artificial human milk containing a slightly higher level of protein promoted considerably better growth than was obtained on the human colostrum.

The failure of rats to grow on mature human milk is probably due to: (1) insufficient protein, more particularly protein containing adequate methionine; (2) a deficiency of the unidentified factor S; and (3) a level of lactose in excess of that which can be tolerated by the rat.

Human colostrum is probably deficient in protein, more specifically the amino acid methionine, and contains too much

lactose for the rat, but does not appear to be deficient in factor S.

While the rat is obviously a poor experimental animal for use in attempting to evaluate a food for young infants, the results presented here show that, at least as far as the rat is concerned, the artificial human milk formulated to contain a gross composition similar to that of average human colostrum is as nutriitous, if not more so, than the human colostrum used in these studies.

ACKNOWLEDGMENT

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SOME PROPERTIES OF AN UNIDENTIFIED GROWTH FACTOR PRESENT IN FISH PRODUCTS [1]

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Many reports in the literature have described an unidentified factor or factors present in certain animal products and required for optimum chick growth. Attempts to concentrate and isolate the factor by different investigators (Johnson et al., '42; Richardson et al., '42; Evans, Carver and Hamm, '44; Scott, Norris and Heuser, '46; Jaffee and Elvehjem, '47; McGinnis et al., '47; Nichol and associates, '47; Robblee et al., '48; and Essary and associates, '48) have disclosed some of its properties. The following properties appear to be agreed upon by more than one laboratory. It is soluble in water and in various concentrations of ethanol and methanol; insoluble in acetone; thermostabile; adsorbed by Fuller's earth and Norit A and eluted by an ammonia-ethanol mixture; and dialyzable through a cellophane membrane.

The following experiments were conducted to obtain further information concerning the properties of the unidentified factor or factors present in sardine fish meal and condensed fish solubles.

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EXPERIMENTAL

Sardine fish meal and condensed sardine fish solubles were the basic materials for the preparations as is indicated in table 1.

Extraction with ethanol

A quantity of sardine fish meal was exhaustively extracted with ethyl ether, dried, and refluxed with 80% ethanol for 5 hours. The mixture was filtered and a fresh quantity of 80% ethanol added. This refluxing process was repeated 4 times, for a total of 20 hours of refluxing. The combined filtrates were consolidated and the ethanol removed by distillation under reduced pressure. The extract was adjusted to pH 4.5 with HCl to prevent decomposition. The extracted sardine fish meal was dried at 80°C.

Enough 95% ethanol was added rapidly to a quantity of condensed fish solubles to form an 80% ethanol solution. This solution was stirred for 30 minutes and filtered. The precipitate was resuspended in a fresh quantity of 80% ethanol, stirred for 30 minutes and filtered. The precipitate was washed with a small quantity of 80% ethanol and dried at 80°C. The filtrates were combined and the ethanol removed by distillation under reduced pressure. The remaining concentrated solution was adjusted to pH 4.5 with HCl.

Adsorption with Darco G-60

A quantity of Darco G-60, equivalent by weight to twice the solids present in the extract, was added to a water or 80% ethanol solution of the active material extracted by 80% ethanol from sardine fish meal or condensed fish solubles, adjusted to pH 3.0 with HCl. The mixture was stirred for 30 minutes and filtered. The process was repeated on the filtrate twice more with fresh amounts of Darco G-60, one-half as much the second time and one-quarter as much the third time. The non-adsorbed filtrates were consolidated and concentrated at room temperature before a fan. The Darco G-60

portions were combined and stirred for 30 minutes with an equivalent quantity by weight of 10% ammonium hydroxide in 95% ethanol. The mixture was filtered and the residue treated twice more with similar fresh quantities of the eluting mixture. The filtrates were consolidated and concentrated before a fan at room temperature.

Precipitation with 75% acetone

A water solution of the adsorbed fraction of an 80% ethanol extract of sardine fish meal was poured with stirring into 100% cold acetone to form a 75% acetone solution. The mixture was refrigerated at −10°C. for two hours, filtered, and the precipitate washed with small quantities of cold acetone. The acetone was removed from the filtrate by concentrating before a fan at room temperature. The precipitate was redissolved in distilled water.

Precipitation with $(NH_4)_2SO_4$

A solution of adsorbed 80% ethanol soluble condensed fish solubles was saturated with ammonium sulfate. The mixture was stirred for one hour and filtered. Excess ammonium sulfate was removed from the filtrate and precipitate by precipitation with $Ba(OH)_2$.

Isoelectric precipitation

A solution of 80% ethanol soluble condensed fish solubles was adjusted to pH 3.0 with HCl, and filtered. The precipitate was washed with small quantities of pH 3.0 water and then redissolved in distilled water at pH 7.0.

Extraction with n-butanol

A solution of 80% ethanol soluble condensed fish solubles was extracted 4 times with equal quantities by volume of *n*-butanol. The *n*-butanol was removed by concentration before a fan at room temperature and the extracted material redissolved in pH 7.0 water.

Testing of preparations

Chick feeding experiments were conducted to test the potency of the various fractions prepared either from sardine fish meal or sardine condensed fish solubles. The experiments consisted of 9 trials with mixed sexes of single-comb White Leghorn chicks hatched from hens that had been on the same all-plant protein ration. The experimental chicks were housed in electrically heated batteries with raised screen floors and for the first two weeks after hatching all chicks were fed the basal diet, which consisted in percentages of yellow corn 43.25; soybean oil meal (mixture of 4 commercial expeller sources) 50.00; dehydrated alfalfa meal (17% protein) 3.00; dried fermentation solubles (500 μg of riboflavin per gram) 0.50; steamed bone meal 1.50; ground limestone 1.00; iodized salt 0.50; and cod liver oil (2000 U.S.P. units of vitamin A and 400 A.O.A.C. units of vitamin D per gram) 0.25. Sixty milligrams of manganese sulfate and 4.50 mg of niacin were added to each pound of this ration. Feed and tap water were supplied ad libitum. At the end of the second week the chicks were leg-banded, weighed, and selected for the experiment. All the very light and very heavy birds were discarded. Those of intermediate weight were divided into groups of 20 chicks according to individual weights, so that each group was balanced with every other group on a weight basis. The chicks were weighed individually at weekly intervals, and at the same time feed consumption was recorded. The duration of the trials was 6 weeks. All preparations were added to the basal ration. The effects of the various fractions of sardine fish meal and condensed fish solubles upon the growth of the chicks are summarized in table 1.

RESULTS AND DISCUSSION

All results are expressed as "response" rather than as average weights to facilitate a direct comparison of the results of different trials. In each trial the unsupplemented basal group and the group fed the basal ration supplemented with

The comparative growth response of chicks fed fish product fractions at levels equivalent to in positive control ration, or as indicated

PREPARATION	ADDED SOLIDS OF PPTN. IN RATION [1]	RESPONSE EXPRESSED AS PER CENT OF IN GROWTH BETWEEN POSITIVE AND CONTROL GROUPS [2]					
		Trial					
		1	2	3	4	5	6
	%						
(1) Fish meal insoluble in 80% ethanol	2.217	—5	—2				
(2) Fish meal soluble in 80% ethanol	0.180	81	66				
(3) Fish meal soluble in 80% ethanol ≃ 6%	0.382	92	99	124	76	98	56
Adsorbed by Darco G-60 at pH 3 from 80% ethanol solution of (3) ≃ 6%	0.066			35	28	41	
Non-adsorbed by Darco G-60 at pH 3 from 80% ethanol solution of (3) ≃ 6%	0.183			106	66	96	
Non-adsorbed by Darco G-60 at pH 3 from a water solution of (3) ≃ 6%	0.119	—2	14			—1	
(4) Adsorbed by Darco G-60 at pH 3 from a water solution of (3) ≃ 6%	0.132	81	92			95	89
75% acetone-soluble of (4) ≃ 6%	0.119	92	79				
75% acetone-insoluble of (4) ≃ 6%	0.027	63	54				
Adsorbed by Darco G-60 from 80% ethanol solution of (4) ≃ 6%	0.022						—20
Non-adsorbed by Darco G-60 from 80% ethanol solution of (4) ≃ 6%	0.070						24
(5) Fish solubles adsorbed by Darco G-60 at pH 3	0.199						
(6) Fish solubles non-adsorbed by Darco G-60 at pH 3	0.330						
(7) Fish solubles insoluble in 80% ethanol	0.440						
(8) Fish solubles soluble in 80% ethanol	0.417					84	
Non-adsorbed by Darco G-60 from water solution of (8)	0.173					—19	
(9) Adsorbed by Darco G-60 from water solution of (8)	0.168					76	
Adsorbed by Darco G-60 from 80% ethanol solution of (9) ≃ 4%	0.054						
Non-adsorbed by Darco G-60 from 80% ethanol solution of (9) ≃ 4%	0.048						
n-butanol soluble of (9) ≃ 4%	0.036						
n-butanol insoluble of (9) ≃ 4%	0.153						
pH 3 soluble of (9) ≃ 4%	0.180						
pH 3 insoluble of (9) ≃ 4%	0.020						
Saturated ammonium sulfate-soluble of (9) ≃ 4%	0.091						
Saturated ammonium sulfate-insoluble of (9) ≃ 4%	0.112						

[1] PPtn = preparation.
[2] Level of fish product in positive control ration: Sardine fish meal, 3%; fish solubles, 2

3% sardine fish meal or 2% condensed fish solubles serve as negative and positive controls. The per cent response is calculated by considering the difference between the negative and positive controls as 100%. A response of zero indicates that the growth of chicks fed the supplemented ration was the same as that with the basal ration, while a response of 100 indicates a growth equivalent to that on the original fish product supplemented ration.

The growth factor, or factors, was extracted from both the sardine fish meal and the condensed fish solubles by 80% ethanol. The factor was soluble in 75% acetone but insoluble in *n*-butanol. Most investigators have stated that the growth factor is insoluble in acetone, whereas the growth results presented in table 1 show that the factor was soluble under the conditions of the present experiments. This is in agreement with Bird et al. ('48), who observed that the growth factor present in cow manure was soluble in 80% acetone.

The activated charcoal, Darco G-60, adsorbed the activity at pH 3.0 from a water solution of an 80% ethanol extract of sardine fish meal or condensed fish solubles. This activity could easily be eluted with 10% ammonium hydroxide in 95% ethanol, with but 20% loss of activity. McGinnis et al. ('47), on the other hand, reported that the factor was not adsorbed by activated charcoal at pH values from 2.0 to 5.0. Their results may indicate a possibility that more than one factor is involved, but a single factor could be responsible for the results noted since their experiments were carried out under different conditions and with liver preparations. In addition, in the experiments reported in this paper Darco G-60 was unable to adsorb the factor from an 80% ethanol soluton. The pigments present in the crude extracts were adsorbed, leaving the active material in a colorless filtrate.

Precipitation of inert material was obtained by adjusting the pH of the solution to 3.0. The growth-promoting substance remained in solution. Saturated ammonium sulfate precipitated the active material, but there is no definite information

concerning the association of the factor with protein in the ammonium sulfate precipitate.

The unidentified factor or factors in fish products examined in these studies exhibited "animal protein factor" activity and properties similar to those reported by other investigators. Recently Ott, Rickes and Wood ('48) reported that crystalline vitamin B_{12} elicited growth response in chicks comparable to that obtained with crude sources of the animal protein factor, indicating that vitamin B_{12} is identical with or closely related to this factor. Further work will be required to determine whether the growth factor, or factors, in fish products is identical with vitamin B_{12} or comprises a combination of this vitamin and a closely related factor.

SUMMARY

The essential chick growth factor present in sardine fish meal and condensed fish solubles was soluble in 75% acetone and in 80% ethanol. It was insoluble in *n*-butanol. The factor was adsorbed by Darco G-60 at pH 3.0 from a water solution of an alcoholic extract of sardine fish meal or condensed fish solubles, but not adsorbed from an 80% ethanol solution of the same extract. Elution of the factor from Darco G-60 can be accomplished with 10% ammonium hydroxide in 95% ethanol with about 20% loss of activity. The factor was colorless in solution.

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THE PANTOTHENIC ACID REQUIREMENTS OF CHICKS RECEIVING A PURIFIED DIET[1]

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ONE FIGURE

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Although there have been several reports relating to the subject, the pantothenic requirement of the chick is still debatable. The original figure of 1400 μg per 100 gm of diet found by Jukes ('39) was approximately twice that reported by Baurenfeind and co-workers ('42). More recently Lepkovsky et al. ('45) report that somewhere between 460 and 970 μg per 100 gm of diet are required. The same authors estimate that the turkey poult has a somewhat higher requirement, between 970 and 1170. Hegsted and Perry ('48) have estimated the requirement of ducklings to be approximately 1100 μg per 100 gm of diet.

The recent findings of Lipmann and co-workers ('47b) offer a possible explanation for the discrepancies among these studies. They have shown that much of the pantothenic acid in foods is combined in the form of the more complex compound designated coenzyme A, the pantothenic acid of which is unavailable to *L. casei* by the usual methods of microbiological assay. Hegsted and Lipmann ('48) showed that the pantothenic acid in coenzyme A is also only partially

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available to the chick. The over-all average of several tests indicated a mean availability of only 66% of the total pantothenic acid in the compound. It seems likely, therefore, that the different basal diets used in the several studies on pantothenic acid requirements may have varied in pantothenic acid content as well as having had various degrees of availability. In this regard it is of interest that the difference in the results obtained by Lepkovsky et al. ('45) with two different diets may be accounted for principally by the difference in the pantothenic acid content of the diets as determined microbiologically.

We report in this paper a study of the pantothenic acid requirements of chicks fed a purified diet rather than the heated diets previously used. Since the basal diet probably contains only an insignificant amount of pantothenic acid, the results should not be complicated by the presence of combined pantothenic acid of an unknown degree of availability. Also the results should be comparable to those obtained in the previous study upon the pantothenic acid requirements of ducklings (Hegsted and Perry, '48), in which a similar diet was used.

EXPERIMENTAL

The pantothenic acid low diet was the same as that described by Hegsted and Perry ('48) except that the corn oil was reduced by 5% and the glucose increased by this amount. A total of 126 White Leghorn chicks were used. They received a standard chick mash until they were one week old and were then divided into 21 groups of 6 chicks each and given a pantothenic acid low diet for 5 days. After this partial depletion they were given the same diet to which various levels of calcium pantothenate had been added. Two groups (6 per group; 12 chicks per level) received diets containing 130, 300, and 500 µg per 100 gm of diet and three groups (6 per group; 18 chicks per level) received diets containing 700, 1000, 1500, 2500, and 4500 µg per 100 gm of diet. The chicks were weighed thrice weekly for three weeks. When they were

killed, the livers from each group were removed immediately, weighed, homogenized together in water, and the suspension heated to boiling. An aliquot was frozen and later analyzed microbiologically for total pantothenic acid, utilizing pigeon liver extract and phosphatase to free the bound acid, since this has been shown to release the total bound vitamin (Lipmann, Kaplan and Novelli, '47a).

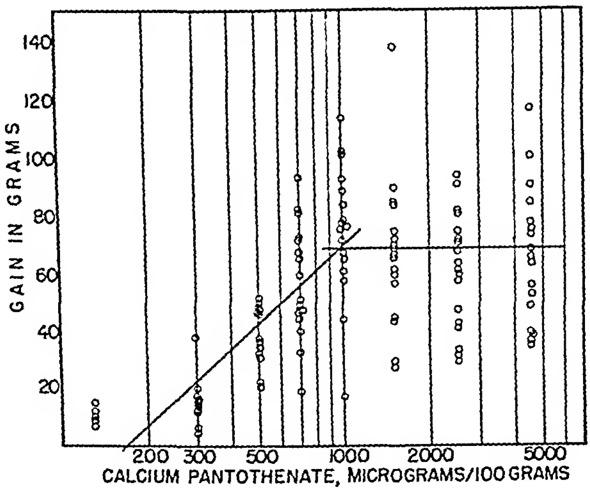

Fig. 1 Scatter diagram showing the relation between the calcium pantothenate content of the diet and the weight gain during the last 10 days of the experiment.

RESULTS

In order to minimize the effects of the previous depletion or of storage of pantothenic acid, only the gains during the last 10 days on the experiment were used. These data were analyzed as previously described (Hegsted, '48) by calculating the regression line shown in figure 1. The 130 µg level was clearly less than the maintenance requirement, since 7 of the 12 birds failed to survive, and these data were not included in calculating the line. The gains from the 1500 µg level and above were also excluded, since these appear to be above the region of linearity. The regression line bisects the abscissa

at 171 µg of calcium pantothenate per 100 gm of diet (156 µg of pantothenic acid), which is assumed to be the best estimate of the maintenance requirement. To allow a gain of 68 gm, which was the mean gain of all the chicks which received more than 1000 µg per 100 gm of diet, approximately 955 µg of calcium pantothenate (874 µg of pantothenic acid) per 100 gm of diet were required. This has been taken as the best esti-

TABLE 1

The relation between the pantothenic acid level of the diet and the liver concentration of pantothenic acid

GROUP NUMBER	DIETARY Ca PANTOTHENATE	NUMBER OF LIVERS IN SAMPLE	MEAN WEIGHT OF CHICKS[1]	MEAN WEIGHT OF LIVERS	PANTOTHENIC ACID CONCENTRATION IN LIVERS
	µg/100 gm		*gm*	*gm*	*µg/mg*
1	130	1	108	4.3	40
2	130	4	93	3.5	50
3	300	6	96	4.3	56
4	300	4	98	4.3	25
5	500	5	116	5.4	71
6	500	6	122	5.6	66
7	700	6	155	7.4	62
8	700	6	145	5.9	79
9	700	5	137	5.6	54
10	1000	6	170	6.1	71
11	1000	6	193	7.7	79
12	1000	6	177	8.0	57
13	1500	3	165	6.0	68
14	1500	3	198	6.9	77
15	1500	3	148	5.7	81
16	2500	3	180	5.9	66
17	2500	3	154	5.3	74
18	2500	3	190	7.8	54
19	4500	3	167	5.3	73
20	4500	3	148	5.2	55
21	4500	3	200	7.0	80

[1] Only three chicks from each group were taken from groups 13–21. The weight shown for these groups is that of the chicks whose livers were analyzed rather than the group mean.

mate of the mean pantothenic acid requirement for growth under these conditions. It is obvious from the wide scatter of the data that the estimate is not particularly reliable.

The pantothenic acid content of the livers was of considerable interest. As may be seen from table 1, the concentration of pantothenic acid was less than normal only in the groups which received 130 and 300 μg of calcium pantothenate per 100 gm of diet. The next higher levels allowed apparently normal liver concentrations, although growth was depressed. It therefore appears that liver concentration is not a sensitive criterion of the adequacy of the diet, at least much less so than growth. A calculation of the total liver content of pantothenic acid showed a much better correlation with growth, but this was primarily a matter of liver size rather than liver concentration.

DISCUSSION

As indicated in the introduction to the present paper, one of the possible explanations for the variations in the estimates of the pantothenic acid requirement of chicks may lie in the fact that heated diets have been used which may contain unknown amounts of pantothenic acid and which may be utilized to an unknown extent. The present estimate of 900 μg per 100 gm of diet is, however, in the same range as the higher estimate made by Lepkovsky et al. ('45). It is somewhat, but probably not significantly, less than the previous figure found for the duckling using the same diet, which was 1100 μg per 100 gm of diet. As suggested by Lepkovsky and co-workers, the rate of growth may be related to the pantothenic acid needs and this may account for the higher figures obtained for ducklings and turkey poults.

The failure of the concentration of pantothenic acid in the liver to be directly related to the pantothenic acid fed, and the finding that it was a less sensitive measure of the adequacy of the diet than was the rate of growth, was somewhat unexpected since tissue analyses for several of the vitamins are thought to be rather precise measures of dietary deficiency.

THE BIOLOGICAL AVAILABILITY OF THE CALCIUM IN BONE

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The recommended dietary allowances for calcium range from 1 gm per day for infants to 1.3–1.4 gm per day for adolescents. The allowance for adults has been set at 1 gm per day. This is increased to 1.5 gm per day during the latter part of pregnancy and 2 gm during lactation (Food and Nutrition Board, N.R.C., '48).

Present human dietaries are largely dependent on milk and milk products to supply the bulk of this calcium. This, however, was obviously not the case centuries ago, as man in the past, in common with other carnivora, was dependent on animal and fish bones for a large portion of his calcium intake. Today many aboriginal people still depend on bone as their chief source of calcium. Bone also supplies many other minerals essential for normal nutrition.

In many areas of the world the supply of milk and milk products is not sufficient to provide the recommended dietary allowances for calcium. During and immediately after the last war a large proportion of the canned meat products prepared in Canada for U.N.R.R.A. contained 15% of cooked ground bone;[1] this resulted in the final canned meat containing approximately 0.8 gm of calcium per 100 gm. Today

[1] "Cooked ground bone" was prepared commercially by cooking whole fresh bones under steam pressure until soft and then grinding the product.

The calcium supplements consisted of 50 gm of dried skim milk per subject per day (reconstituted with 150 ml of water) or 2.15 gm of bone meal. In either case the calcium supplied by the supplement was 0.646 gm per person per day. The basal diet contributed an average of 0.218 gm of calcium per day, making a total intake of 0.864 gm per day. The subjects were divided into two groups of three each. The milk or bone meal supplements were alternated between the groups and were changed for successive collection periods. That is, group 1 received a bone meal supplement for the first and third collection period and milk for the second and 4th period, while group 2 received a milk supplement for the first and third period and bone meal for the second and 4th. By planning the experiment in this way a measure of compensation was obtained for variations in the mean calcium balance that might be caused by changes in the basal menu or in the basal calcium intake. Carmine was used as a feces marker. No preliminary adjustment period was considered necessary as the basal diet plus supplement supplied a level of calcium to which the majority of the subjects was normally accustomed.

RESULTS

The experimental results are recorded in table 1. The mean balance when the calcium supplement was supplied as milk was —47 mg per day. The balance was —73 mg per day when the calcium supplement was supplied as bone meal. This loss in balance of 26 mg per day would seem to indicate a somewhat decreased availability of the calcium in bone meal compared to that in milk. However, in view of the variations in the individual results, little signficance can be attached to this observation. Statistical analysis showed this to be true, the standard error of the difference between means proving to be ± 34 mg.

A disturbing feature about these results is the negative balances observed at a calcium intake level that is usually considered adequate to maintain calcium equilibrium. Steg-

all flour sold in Newfoundland, where the consumption of milk is small, contains ½ of 1% bone meal.[2] For many years bone meal has been an ingredient of certain infant cereal products.[3] Because of this use of bone, the question of the availability for humans of the calcium from this source has assumed considerable importance. It was for this reason that the present study of the comparative availability of the calcium in bone and in milk was undertaken.

EXPERIMENTAL

The experiment was carried out according to the following plan. The entire study was divided into 4 successive collection periods of 5 days each. Six subjects (4 women and two men) undertook the experimental regime. The ages of the women were 20, 24, 29 and 32, while the men were 38 and 56 years old. The experiment took place in April, so there was no excessive loss of calcium in perspiration. All subjects ate a low calcium diet devised and prepared by the Hospital for Sick Children diet kitchen and served in the staff dining-room. All portions were carefully weighed and were completely consumed by the subjects. A 7th identical portion of each item was prepared and reserved for calcium analysis. The sample meals were pooled for each collection period and the amount of calcium in the pooled sample determined. All water used in cooking and for drinking was distilled, as Toronto city water contains an appreciable amount of calcium. The menu for breakfast was unchanged throughout the study and the calcium supplement was taken at this meal. Breakfast consisted of fruit juice, cream of wheat porridge with corn syrup, white toast, butter, honey and coffee. A capsule supplying 5000 units of vitamin A and 700 units of vitamin D was taken at this meal. The menu of the other meals was varied from day to day.

[2] "Bone meal" refers to the fine dry powder obtained by boiling the bones to remove fat and a good deal of organic material, drying with hot air, and grinding.

[3] Pablum, Pabena.

The calcium supplements consisted of 50 gm of dried skim milk per subject per day (reconstituted with 150 ml of water) or 2.15 gm of bone meal. In either case the calcium supplied by the supplement was 0.646 gm per person per day. The basal diet contributed an average of 0.218 gm of calcium per day, making a total intake of 0.864 gm per day. The subjects were divided into two groups of three each. The milk or bone meal supplements were alternated between the groups and were changed for successive collection periods. That is, group 1 received a bone meal supplement for the first and third collection period and milk for the second and 4th period, while group 2 received a milk supplement for the first and third period and bone meal for the second and 4th. By planning the experiment in this way a measure of compensation was obtained for variations in the mean calcium balance that might be caused by changes in the basal menu or in the basal calcium intake. Carmine was used as a feces marker. No preliminary adjustment period was considered necessary as the basal diet plus supplement supplied a level of calcium to which the majority of the subjects was normally accustomed.

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gerda and Mitchell ('46) give an average daily requirement compiled from the literature of 653 mg. In the same report a mean balance of —125 mg per day for a mean low calcium intake of 222 mg per day was reported. This was derived

TABLE 1

Calcium balances with milk or bone meal

COLLECTION PERIOD	SUPPLEMENT TO LOW Ca DIET	Ca IN BASAL DIET	TOTAL Ca INTAKE PER DAY	Ca IN URINE PER DAY	Ca IN FECES PER DAY	BALANCE
		mg	*mg*	*mg*	*mg*	*mg*
	Subject M.W.					
3	Bone meal	235	881	131	693	+ 57
4	Milk	206	852	153	703	— 4
	Subject N.J.					
1	Bone meal	215	861	158	710	— 6
2	Milk	216	863	165	648	+ 50
3	Bone meal	235	881	168	752	— 39
4	Milk	206	852	170	652	+ 30
	Subject D.D.					
1	Bone meal	215	861	132	790	— 60
2	Milk	216	863	152	628	+ 83
3	Bone meal	235	881	129	1036	—284
4	Milk	206	852	177	768	— 92
	Subject R.P.					
1	Milk	215	861	152	630	+ 79
2	Bone meal	216	863	120	792	— 49
3	Milk	235	881	137	628	+116
4	Bone meal	206	852	107	507	+238
	Subject S.J.					
1	Milk	215	861	159	800	— 97
2	Bone meal	216	863	146	778	— 61
3	Milk	235	881	159	815	— 93
4	Bone meal	206	852	127	792	— 67
	Subject T.D.					
1	Milk	215	861	178	860	—177
2	Bone meal	216	863	195	825	—157
3	Milk	235	881	194	1105	—418
4	Bone meal	206	852	222	960	—330

Mean balance for milk supplement = — 47 mg.
Mean balance for bone meal supplement = — 73 mg.
Difference of means = — 26 mg.

from data obtained from 19 subjects observed for a total of 920 man days. If this balance is assumed to apply to the present group, the utilization of the calcium in the milk supplement is only 12.1%, while that of the bone meal is 8.75%. Steggerda and Mitchell ('46) report utilizations for the calcium of milk of the order of 30% when the total calcium intake averaged 545 mg per day. While the higher total calcium intake (864 mg) in the present case would result in a decreased percentage utilization, it would not be expected to depress it to this extent.

Subject T.D. was in consistent gross negative balance throughout the experiment. This subject was accustomed to a high calcium intake in his normal diet. Furthermore, the feces were diarrheal in nature, being loose and unformed for the whole period. These two features may well account for the negative balances in this case. If the results for this subject are disregarded, the mean balance for the milk supplement becomes + 8 mg per day, and the percentage utilization becomes 20.7%. These values are both in the expected range. The balance with the bone meal supplement is then — 30 mg per day and the utilization is 14.8%.

If we separate the individual subjects into two groups, first, those who exhibited a better calcium balance when receiving bone meal, and, secondly, those who exhibited a better calcium balance when receiving milk, we find that subjects M.W., S.J. and T.D. all showed better utilization of the calcium in bone meal, while subjects N.J. and D.D. utilized the calcium of milk better than that of bone meal. Subject R.P. does not fall into either group, as the mean balance of + 98 for the two periods with milk is almost identical with the mean balance of + 95 with bone meal. By this method of comparison the availability of the calcium in bone meal would appear to be as good as that of milk. In any case, an accurate figure for the relative availability of the calcium in bone meal and in milk cannot be obtained until such time as the results of sufficient studies of this nature to warrant a statistical analysis have been published. This study indicates only that

the availability of the calcium in bone meal is comparable to that of milk.

The utilization of calcium by the rat

A variety of cooked ground bones had been prepared by Canada Packers, Limited, at the request of the authors. Experiments were set up to determine the retention of calcium from these bones by the rat. A sample of the bone meal used in the human study was included. Whole dried milk powder and calcium carbonate were used as controls. Six rats, about 6 weeks old and 50 gm in weight, were placed in each of 6 groups. The groups were matched for litter and sex. Each group was placed on a low calcium diet to which was added 0.200% calcium in the form of the substance to be tested. The Chant Robertson low mineral diet (Robertson and Doyle, '35) was used with the addition of 1.8% of Osborne and Mendel's salt mixture without the calcium salts. The diets were fed ad libitum for a period of 4 weeks, records being kept of the amount of food consumed. The rats were then killed, the gastrointestinal tracts removed, and the carcasses autoclaved in 3% acetic acid at 15 pounds steam pressure for two hours. This so softened the bones and teeth that they were readily homogenized in a Waring Blendor. The carcasses from each group were pooled and thoroughly homogenized and a sample removed for calcium analysis (Tisdall and Drake, '38).

The results are recorded in table 2. The first column lists the substances supplying the calcium supplement. The second and third columns give the total amount of calcium consumed and that supplied by the supplement only. The next two columns give the total calcium and phosphorus found in the rat carcass at the end of the feeding period. The next column illustrates that, within narrow limits, the phosphorus retention is proportional to the calcium retained. The 7th column gives the extra calcium retained over and above that in the low-calcium group. The percentage retention in the last column is calculated from the data in columns 3 and 7.

TABLE 2

The retention of calcium by rats

	Calcium supplement	Total Ca consumed	Ca supplied by supplement	Average Ca in rats [1]	Average P in rats [1]	Ratio Ca/P	Additional Ca retained by supplemented animals	Supplementary Ca retained
		mg	*mg*	*mg*	*mg*		*mg*	%
Run no. 1	Whole milk	557	526	934	689	1.36	483	91.9
	Hard pork and beef bones	547	516	891	635	1.43	441	85.4
	Soft pork and beef bones	556	525	872	650	1.34	421	80.2
	Straight run bones	555	524	936	706	1.33	485	92.6
	$CaCO_3$	599	568	908	687	1.32	457	80.5
	Low Ca diet	31		451	408	1.10		
Run no. 2	Low Ca diet	30		488	453	1.08		
	Whole milk	631	601	1054	856	1.23	566	94.2
	Lunch meat + 12% bone	733	703	1086	832	1.31	598	85.1
	Dried bone meal	641	611	996	783	1.27	508	83.2
	Cooked bone (U.N.R.R.A.)[2]	613	583	915	750	1.22	427	73.3
	$CaCO_3$	642	607	1032	744	1.39	477	78.6

[1] Six rats in each group.

[2] Prepared commercially by cooking whole fresh bones under steam pressure until soft and then grinding the product. This material was used in meat products prepared in Canada for U.N.R.R.A., as mentioned in the text.

There appears to be no great difference in the retention of calcium from any of the bone products. The mean retention for all bone products was 83.3%, compared to an average retention of calcium from whole dried milk of 93%. Thus the retention of the calcium of bone is about 90% of that of milk for young growing rats. This is in the same range as the utilization found in the human study and tends to corroborate that result.

SUMMARY

A comparison has been made of the utilization by humans of the calcium of skim milk powder and of bone meal.

Six subjects received a low calcium diet for four 5-day periods. Supplements of skim milk powder or bone meal supplying the same amounts of additional calcium were given during alternate collection periods.

The availability of the calcium in bone meal appeared to be in the same range as that of milk.

A study of the retention by young rats of calcium from whole cooked ground bone is also reported.

The retention of calcium from this source was approximately 90% of the retention from whole dried milk.

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THE SULFUR AMINO ACID REQUIREMENTS OF TURKEY POULTS

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TWO FIGURES

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The importance of methionine and cystine in rations for growing chicks has been investigated extensively (Almquist, '47), but the need for these amino acids by growing poults has not been reported on. By employing an isolated soybean protein as the principal source of amino acids it was possible to devise a basal ration in which the normal growth of poults was limited by methionine and cystine deficiencies. By varying the cystine and methionine additions to the basal ration it was possible to determine the amounts of these amino acids required for optimum growth.

EXPERIMENTAL

The basal ration contained, per 100 gm: isolated soybean protein,[1] 28.0 gm; calcium gluconate, 5.0 gm; cellulose,[2] 5.0 gm; crude soybean oil, 3.0 gm; salt mixture, 2.5 gm; vitamin mixture, 2.0 gm; tricalcium phosphate, 2.0 gm; condensed fish solubles,[3] 2.0 gm; dicalcium phosphate, 1.5 gm; fish oil (1000 A, 400 D), 0.5 gm; choline chloride, 0.3 gm; cholic acid, 0.1 gm; inositol, 0.1 gm; and alpha-tocopherol acetate, 2 mg. The bal-

[1] "Alpha protein" from the Glidden Company.

[2] "Cellu flour."

[3] Kindly donated by Valley Dehydrating Company, Kingburg, California, through the courtesy of Mr. E. B. Fischel.

ance of the ration was supplied by cornstarch. The mineral mixture supplied the following in grams per 100 gm of ration: sodium chloride, 1.0; dipotassium phosphate, 0.5; magnesium sulfate, 0.3; potassium chloride, 0.3; sodium silicate, 0.2; manganese sulfate, 0.03; aluminum sulfate, 0.025; ferric oxide, 0.02; copper sulfate, 0.005; zinc sulphate, 0.005; cobalt acetate, 0.002; and potassium iodide, 0.001 gm. The vitamin mixture supplied the following in milligrams per 100 gm of ration: riboflavin, 1.0; thiamine hydrochloride, 1.0; pyridoxine hydrochloride, 1.0; calcium *(d)* pantothenate, 3.0; nicotinic acid, 10.0; pteroylglutamic acid,[4] 0.5; 2-methyl-1,4-naphthoquinone, 1.0; and biotin,[5] 0.02.

Bronze poults were fed a practical starting ration for from 6 to 12 days after hatching. They were then distributed into groups of equivalent weight and were fed the experimental rations. There were from 5 to 10 poults per group in experiments in which the levels of methionine were varied, and 9 or 10 per group in experiments in which the levels of cystine were varied. The poults were housed in electrically heated batteries with raised wire floors. The feed and water were supplied ad libitum. The experiments were continued from 9 to 14 days and the birds were weighed and examined at frequent intervals.

The isolated soybean protein was found to contain 82.6% crude protein, 1.30% methionine and 0.17% cystine.[6] The methionine content was determined by the method of McCarthy and Sullivan ('41) as modified by Grau and Almquist ('45), and cystine was measured by the method of Rossouw and Wilken-Jorden ('35) as modified by Sullivan and Hess ('37). The condensed fish solubles contained 30% crude protein and were assumed to supply to the ration 0.009%

[4] Kindly donated by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York, through the courtesy of Dr. T. H. Jukes.

[5] Kindly donated by Merck and Company, Rahway, New Jersey, through the courtesy of Dr. D. F. Green.

[6] We are indebted to Miss M. Kamei of the Division of Poultry Husbandry, University of California, Berkeley, for making this determination.

methionine and 0.003% cystine, according to the values given by Lassen and Bacon ('46). By calculation on the basis of the above values, the basal ration contained 23.7% crude protein, 0.37% methionine and 0.05% cystine.

The DL-methionine [7] and L-cystine used in these studies were commercial preparations.

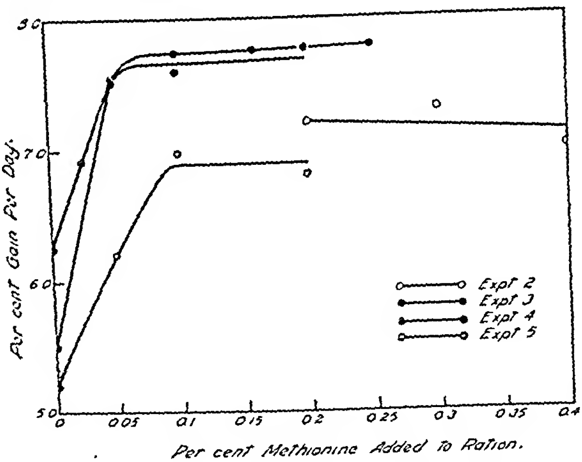

Fig. 1 The relation of the growth of turkey poults to the level of methionine added to a ration containing adequate cystine (0.55%). Notice that with levels of methionine greater than 0.1% there was no further increase in growth. The addition of cystine alone to the basal diet produced gains of only 5.2% to 6.2%.

RESULTS

When the basal ration was fed without the addition of either cystine or methionine, the gain of poults was only 0.8% per day,[8] compared with 7.0% for poults fed the same ration adequately supplemented with the sulfur amino acids. The addition of cystine to the ration produced a gain of 5.2% per day, indicating that cystine alone does not produce optimum growth.

In order to insure an excess of cystine in the ration, 0.5% cystine was arbitrarily added to the basal ration in the next

[7] See footnote 5, p. 378.

[8] $$\text{Per cent gain per day} = \frac{\text{Average gain} \times 100}{\text{Average weight during experiment} \times \text{no. of days}}.$$

series of trials (experiments 2–5). Various levels of methionine were added to this ration to determine the amount of methionine needed in the presence of adequate cystine. The results are shown in figure 1. Growth increased as the amount of methionine in the ration was increased until a level of between 0.05 and 0.10% methionine was reached. No significant increase was noted above 0.10%.

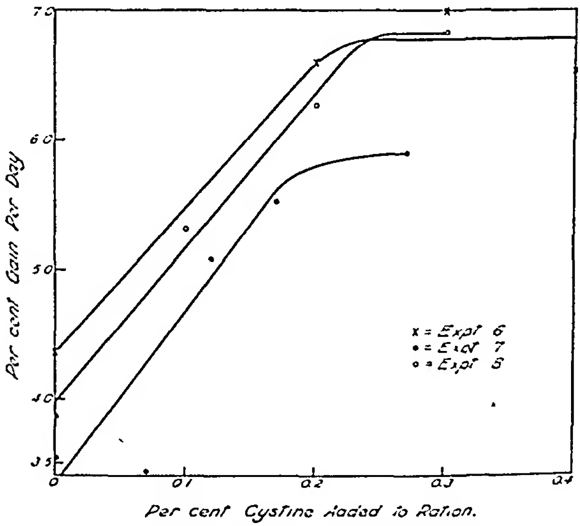

Fig. 2 The relation of the growth of turkey poults to the level of cystine added to a ration containing barely adequate methionine (0.47%). Notice that with a cystine level of about 0.25% there was no further increase in growth.

In another series of tests (experiments 6–8), 0.1% methionine was added to the ration and the level of cystine was varied. In this manner the minimum requirement for methionine was satisfied and cystine was the only factor which limited growth. The results are shown in figure 2. Growth increased as the cystine in the ration was increased until a level of approxmately 0.25% was reached.

Levels of methionine above the minimum level required for growth in the presence of cystine were included in two experiments to test the supposition that methionine can replace cystine in the ration. The pertinent data from these trials

are summarized in table 1. In the first experiment the ration containing 0.4% methionine supported growth as well as the one with adequate amounts of both cystine and methionine. In trial B, 0.3% of methionine was as effective as 0.1% methionine and 0.2% cystine.

TABLE 1

Gains of poults fed rations containing varying amounts of cystine and methionine

SUPPLEMENTS	LEVEL	MILLIMOLES S ADDED PER 100 GM RATION THROUGH Cystine	Methionine	Total	GAIN PER DAY[1]
	%				%
Trial A					
Cystine	0.5	4.15		4.15	5.0
Cystine Methionine	0.5 0.25	4.15	1.68	5.83	6.8
Methionine	0.4		2.68	2.68	6.7
Trial B					
Methionine	0.1		0.67	0.67	3.9
Methionine Cystine	0.1 0.2	1.66	0.67	2.33	6.3
Methionine Cystine	0.1 0.3	2.50	0.67	3.17	6.8
Methionine	0.3		2.01	2.01	6.5

[1] For definition of "gain per day in %" see text.

Poults which were fed the purified ration with adequate supplements of the sulfur amino acids grew as well as poults fed a practical poult starting ration. In 7 experiments in which these comparisons were made, poults fed the practical starting ration grew better three times, poults fed the purified ration did better in two cases, and in two trials there was no difference in growth between birds on the two types of rations.

DISCUSSION

It has been demonstrated that in the chick methionine can be used for at least three distinct functions, namely (1) to meet the requirement for methionine itself, (2) to serve as a precursor of cystine, and (3) to serve as a methylating agent (Almquist and Grau, '45; Almquist, '47; McKittrick, '47). In the present study adequate choline was supplied in all the rations (Evans, '43) to prevent the use of methionine as a methylating agent in choline synthesis.

When 0.5% cystine was added to the ration without additional methionine, suboptimum growth resulted. Optimum growth was obtained with as low as 0.1% methionine and 0.25% cystine. This indicates that in the turkey poult an excess of cystine with adequate choline may not substitute for methionine.

Methionine may be used as a complete source of the sulfur amino acids, in contrast to cystine which will not permit maximum growth when it is the sole supplement. This indicates that in the poult, as in chicks and other animals, methionine is converted to cystine.

The amounts of methionine and cystine required to supplement the basal ration, 0.1% and 0.25% respectively, added to the quantities contained in the basal ration, 0.37% and 0.05% respectively, would place the total amounts required by the poult at approximately 0.5% methionine and 0.3% cystine. This may also be expressed as approximately 0.8% total sulfur amino acids, of which 0.5% must be supplied as methionine.

The sulfur amino acid requirement of the poult, 0.8%, is slightly less than the level of 0.9% which has been reported by Almquist ('47) to be required by the White Leghorn chick. McGinnis and Evans ('47) have reported somewhat lower values for New Hampshire chicks. Comparison of the requirements for poults and chicks is of interest since it has been found that lysine and arginine are required by poults in greater amounts than by chicks (Grau et al., '46; Kratzer

et al., '47). A deficiency of glycine was found to be less critical in the poult than in the chick (Kratzer and Williams, '48).

SUMMARY

Young turkey poults were fed rations containing an isolated soybean protein to which various additions of cystine and methionine were made. Approximately 0.5% methionine and 0.3% cystine were required for the optimum growth of the poults in a ration containing 24% crude protein. Methionine may completely replace cystine in the ration but cystine is not changed to methionine.

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COMPOSITION OF PERCHERON MARES' COLOSTRUM [1]

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It is quite generally recognized that colostrum, secreted during the first days after parturition, differs in many respects from milk produced during the remainder of the lactation period. Of late considerable attention has been devoted to the vitamin content of colostrum. Dan ('33); Henry, Houston and Kon ('40); Luecke, Duncan, Ely, Greene and Tull ('47); Spielman, Thomas, Loosli, Whiting, Norton and Turk ('47); and others have conducted studies of the carotene and vitamin content of colostrum, and Pearson, Darnell and Weir ('46) have determined the amounts of thiamine, riboflavin, nicotinic acid and pantothenic acid present in cow and ewe colostrum. Parrish, Wise and Hughes ('47) concluded that most of the fat-soluble yellow pigment in colostrum was carotene and that practically all of the vitamin A in colostrum was in the ester form. Thomas, Loosli and William ('47), and Spielman, Thomas, Loosli, Whiting, Norton and Turk ('47) established a relationship between the prepartum diet and the amount of carotene and vitamin A in bovine colostrum.

It has been recognized for some time that colostrum is far richer in protein than normal milk. More recently Smith ('46) in his studies of the immune proteins of bovine colostrum has called attention to the important role of colostrum in the transmission of antibodies from mother to offspring, and partic-

[1] Contribution No. 690, Massachusetts Agricultural Experiment Station.

ularly in the protection of the newborn calf against infectious diseases. Also Lewis and Wells ('22) concluded that there is no equivalent substitute for human colostrum for newborn infants and that it furnishes protective antibodies which add much to the newborn infant's capacity to resist infection in early life. But the literature consulted contained very little data regarding the mineral content of colostrum. Luecke, Duncan, Ely, Greene and Tull ('47) found little difference in the iron, copper, and cobalt content of the colostrum of Jersey, Holstein, and beef cows. Garrett and Overman ('40) examined the colostrum produced by purebred Holstein and Ayrshire first-calf heifers and reported that calcium, magnesium, sodium, phosphorus, and chlorine are all high at parturition and during the early hours of lactation and that they decline rather rapidly to a fairly constant level, but that potassium is rather low at parturition and gradually increases to a fairly constant level as the milk progresses toward normality.

Even less information is available regarding the mineral content of mares' colostrum, and this study was undertaken to accumulate some data regarding the mineral content and the protein and reduced ascorbic acid values of colostrum produced by purebred Percheron mares maintained under controlled conditions.

EXPERIMENTAL

The colostrum was obtained from 4 normal, well-developed, healthy Percheron mares. With the exception of mares 2a and 2b, all of the mares were used throughout the larger portion of the year as draft animals on the University farm. The ages, weights and lactation histories of the mares are as follows: Mare 1, 4 years old, 1750 lb., first lactation; mare 2a, 8 years old, 1900 lb., 5th lactation; mare 2b, 10 years old, 1900 lb., 6th lactation; mare 3, 11 years old, 1600 lb., 4th lactation; mare 4, 11 years old, 1800 lb., 5th lactation. Mares 2a and 2b are the same animal.

Colostrum samples were collected at 24-hour intervals, about 8:00 A.M., for the first 6 days of lactation. The initial samples

were obtained from mare 1, before the foal nursed; from mare 2a, about three hours after the foal was born; from mare 2b, early in the morning (the foal arrived at 10:30 P.M.); from mare 3, about 10 hours after foaling; and from mare 4, within an hour after parturition.

The study was conducted during late March and April and early May, and the mares were all confined indoors and fed home-grown, early cut mixed hay ad libitum and three quarts of crushed oats and 5 large ears of dry white dent corn per day. None of the mares had worked for several weeks preceding parturition. The mares and their foals were housed in well-lighted, adequately ventilated, earth floored, box stalls that were approximately 15 ft. square. The mares were well cared for during pregnancy, were disease-free and in excellent physical condition. Hence, the colostrum should have possessed optimum nutritive value for the breed and conditions under which it was produced.

The samples of colostrum were assayed by the recognized methods reported by the Association of Official Agricultural Chemists ('45) for water, protein, reduced ascorbic acid, phosphorus, potassium, and magnesium.

RESULTS AND DISCUSSION

The values obtained from the different assays of colostrum samples for the various constituents are reported in table 1. The amount of water in the colostrum produced by the different mares was fairly constant from day to day, and the average values, 87.5%, 86.3%, 86.3%, 88.1% and 86.3%, indicate considerable uniformity for colostrums from Percheron mares maintained under the conditions of this study. It is interesting to note that identical values were obtained for mares 2a and 2b. As stated above, the designations 2a and 2b are for different lactations of the same animal.

The protein content of the initial sample of colostrum from three of the mares was decidedly higher than for the following days, but the initial sample of colostrum from mare 1 showed less difference in protein content from the subse-

TABLE 1

Composition of Percheron mares' colostrum

MARE NO.	SAMPLE NO.[1]	WATER	PROTEIN[2]	ASCORBIC ACID	PHOS-PHORUS	POTAS-SIUM	MAG-NESIUM
		%	%	mg/l	mg/100 gm	mg/100 gm	mg/100 gm
1	1	85.2	7.7	30	58	74	24
	2	88.0	3.1	52	87	121	12
	3	87.9		80	99	111	14
	4	87.6	3.3	73	87	118	16
	5	88.2	3.2	84	94	86	15
	6	88.6	3.1	79	88	98	14
	Av.	87.5	4.1	66	86	101	16
2a	7	81.0	14.4	33	17	19	11
	8	86.8	3.9	64	78	83	10
	9	87.6	3.3	56	89	98	14
	10	87.3	3.6	55	90	107	14
	11	87.9	3.3	57	87	85	13
	12	87.2	3.6	75	90	87	14
	Av.	86.3	5.4	57	75	80	13
2b	13	82.5	12.1	20	44	27	19
	14	86.7	3.8	45			..
	15	87.0	3.6	91	86	95	16
	16	86.6	3.8	61	93	94	14
	17	87.4	3.4	58	88	96	16
	18	87.6	3.2	52	86	86	14
	Av.	86.3	5.0	55	79	80	16
3	19	87.7	4.0	47	80	112	18
	20	88.2	4.1	58	97	109	15
	21	87.2	3.8	53	88	93	15
	22	88.2	3.8	57	92	108	16
	23	88.4	3.3	60	72	81	14
	24	88.6	3.4	62	83	94	16
	Av.	88.1	3.7	56	85	100	16
4	25	80.1	14.2	32	46	85	26
	26	87.4	3.9	33	110	116	14
	27	86.9	3.6	44	88	94	14
	28	87.4	3.1	61	73	85	10
	29	87.8	3.3	54	94	94	12
	30	88.1	3.1	56	89	92	13
	Av.	86.3	5.2	47	83	94	15

[1] Colostrum samples were collected at 24-hour intervals, about 8:00 A.M., for 6 consecutive days.

[2] Protein = nitrogen × 6.38.

quent samples. Although means of values that vary widely are likely to be of limited service, it may be noted that the mean amount of colostral protein in the 6 samples of colostrum from mare 2a was 5.4% and from mare 2b was 5.0%. Excluding the initial samples, the protein values were very uniform and the mean values were 3.54% for mare 2a and 3.56% for mare 2b, i.e., in successive lactation periods for the same animal. These values are definitely higher than the 2.7%, 2.4%, 2.1%, and 2.0% reported by Holmes, Spelman, Smith and Kuzmeski ('47) for Percheron mares' milk produced between the 12th and 129th days of lactation.

The amount of reduced ascorbic acid in the colostrum of each mare increased during the experimental period. The values for the amounts of ascorbic acid in the 6 samples, as seen in table 1, averaged 66, 57, 55, 56, and 47 mg/l, for mares 1 to 4, respectively. Again the values for mare 2a and 2b are in close agreement. For comparison it may be noted that milk from mares 1, 2 and 4, at a later stage of lactation, was assayed for reduced ascorbic acid. During a 5-week period, 25 samples of milk collected from each mare averaged as follows in milligrams per liter: Mare 1, from the 126th to the 162nd day of lactation, 171; mare 2b, from the 125th to the 161st day of lactation, 146; mare 4, from the 94th to the 130th day of lactation, 134. The higher values for ascorbic acid during the later stages of lactation doubtless were affected by the change in the mares' ration from dry hay and grain to unlimited amounts of grass in a good fresh pasture.

The amount of phosphorus in the initial samples of colostrum was smaller than for the succeeding samples. After the first day the phosphorus content of the colostrum was fairly constant for the individual mares. The average phosphorus contents in mg/l for the 6-day periods were 86, 75, 79, 85 and 83 for mares 1 to 4, inclusive. The values 75 mg and 79 mg per liter were for successive lactations of the same mare (2a and 2b). These values for the phosphorus content of colostrum are higher than the 63 mg per liter value previously reported

by Holmes, Spelman, Smith and Kuzmeski ('47) for normal milk produced by Percheron mares.

The colostrum from mares 1 and 3 contained approximately the same amounts of potassium, the averages being 101 mg and 100 mg per liter. The average potassium content of the colostrum produced by mare 4 was 94 mg per liter, and that for mares 2a and 2b was 80 mg per liter. Judged by these results, colostrum from Percheron mares contains significantly more potassium than their mature milk, for Holmes et al. ('47) reported 64 mg per liter for the latter.

The initial sample of colostrum from 4 of the mares contained more magnesium than the later samples. The respective means for the remaining 5 days of the 6-day periods for the 5 mares, in mg/l, were 16, 13, 16, 16 and 15 for mares 1 to 4, respectively. After the first day of lactation the magnesium content of the mares' colostrum varied from 10 mg per liter for mares 2a and 4 to 16 mg per liter for mares 1, 2b and 3. In general, the results obtained for the individual mares were quite uniform but were higher than those reported in the earlier study of the composition of mares' milk by Holmes and co-workers ('47), who found 9 mg per liter in Percheron mares' milk.

Although mares' colostrum contains two to three times as much protein and definitely more phosphorus, potassium and magnesium than does their later milk, few data are available to indicate whether or not these constituents in the colostrum are equally well or better utilized by the foal than when they occur in normal mares' milk. Frenzel ('37), from his metabolism experiments with young foals, concluded that the digestibility of colostrum during the first week of life is approximately 90%, and that the coefficient of digestibility of the constituents of mares' milk decreases gradually as the foal grows older.

SUMMARY

Thirty samples of colostrum were collected for the first 6 days of lactation from 4 normal, purebred Percheron mares.

Their ages varied from 4 to 11 years, their weights from 1600 pounds to 1900 pounds, and their lactation periods being from the first to the 6th. One mare was used for two lactation periods, her 5th and 6th.

The water content of the colostrum was similar for the different mares. The mean protein values for the 6-day period, which ranged from 3.7% to 5.4%, were markedly higher than the value of 2.3% reported for mature mares' milk.

Relatively small amounts of reduced ascorbic acid were found, i.e., from 47 mg to 66 mg per liter for mares that later produced mature milk which contained from 134 mg to 171 mg per liter.

Initial samples of colostrum contained less phosphorus than the later samples. The mean values in milligrams per liter of 86, 75, 79, 85 and 83, were higher than the 63 mg per liter obtained in an earlier study of mature milk.

The mean values for potassium for the different mares were 101 mg, 80 mg, 80 mg, 100 mg, and 94 mg per liter as compared with 64 mg per liter for mature milk.

The various samples of colostrum contained similar amounts of magnesium. The mean values were significantly higher than those previously obtained for milk produced at the end of the first month of lactation.

Values obtained for colostrum produced during two lactation periods of the same mare were in close agreement.

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THE UTILIZATION OF NICOTINIC ACID BY PREGNANT WOMEN[1]

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TWO FIGURES

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A study of the incidence of pellagra in 7 cotton-mill villages in South Carolina from 1916 to 1920 (Goldberger, Wheeler and Sydenstricker, '20, '28) showed a higher incidence of pellagra among women from 25 to 45 years of age than in any other age and sex group. Kooser and Blankenhorn ('39) found a large proportion of the pellagrins among Kentucky mountain people to be multiparous women. This seems to indicate a greater demand of the maternal body for nicotinic acid during the reproductive cycle. No studies have been reported on the metabolism of this vitamin by pregnant women. The present investigation was undertaken for the purpose of studying the relation of the intake of nicotinic acid to the urinary excretion of some of its metabolites.

EXPERIMENTAL PROCEDURE

Three groups of women were selected for study. Group I[4] included 7 healthy young primigravidae during the last 5

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[4] This study is part of an investigation previously initiated for the purpose of studying the metabolism of riboflavin and thiamine by pregnant women. The riboflavin and thiamine studies were supported by a grant from the American Dairy Association through the National Dairy Council.

months of pregnancy; group II consisted of 42 clinic patients selected at random from a group of women who were serving as subjects in a large clinical study being carried on in the Department of Obstetrics and Gynecology at the University of Chicago [5] (Dieckmann, '48); while group III included 12 non-pregnant women, graduate students in the Department of Home Economics, University of Chicago.

The 7 subjects in group I lived at home and ate self-selected diets. They were selected for study on the basis of (1) freedom from pathological disturbances which might interfere with interpretation of results, (2) willingness to cooperate and (3) accessibility to the laboratory. All except one, subject 6, were clinic patients at the Chicago Lying-in Hospital. Therefore their dietaries were no doubt influenced to some extent by instructions received from the clinic dietitian at the time of their regular clinic visits; otherwise they received no dietary instructions. Subject 6 voluntarily supplemented her diet with 11.2 mg of nicotinamide per day during the last three months of the study, while subject 7 supplemented hers with 20.2 mg of nicotinamide during the entire 5-month period.

Seven-day balance studies were made during each of the last 5 months of pregnancy, including (1) daily nicotinic acid intake, (2) urinary excretion of (a) the acid-hydrolyzable fraction and (b) N^1-methylnicotinamide.

Each subject kept a record of the weights of all foods eaten over the 7-day period, with the close supervision of one of the investigators. Individual food samples for analysis were provided by the subject in closed containers and kept under refrigeration for periods of from three to 4 days. Food composites containing one-fifth of the amount of each food consumed were homogenized in a Waring Blendor and made up to 4 l with 0.15 N HCl. Suitable aliquots were quantitatively transferred into an Erlenmeyer flask and autoclaved at 15 lb pressure (118–120°C.) for 20 minutes and stored in a refrigerator until analyzed.

[5] This study is being carried on in the Chicago Lying-in Hospital.

Twenty-four-hour urine collections were made over the 7-day study period in amber colored bottles containing, as preservatives, glacial acetic acid and toluene. These collections were brought to the laboratory daily and 7-day composites, containing one-tenth of the total daily amount excreted, were made for analysis.

The response to a 50 mg test dose of nicotinamide as measured by the urinary excretion of N^1-methylnicotinamide was determined at the end of each balance study. The N^1-methylnicotinamide excreted during a 4-hour collection period immediately following breakfast on the last day of the balance study served as the control level of excretion. Increased excretion of the methylated derivative after the test dose over a similar 4-hour period on the 9th day of study was used as a criterion for response to the test dose.

The data on the dietary intake of nicotinic acid of the 42 clinic subjects in group II were based upon calculated values of weighed diet records obtained under the supervision of the research dietitian of the Chicago Lying-in Hospital over periods of from 4 to 9 days. The records were carefully discussed with the subjects, and only those women whose dietary records appeared to be complete were included in the study.

The levels of excretion of N^1-methylnicotinamide of this group of subjects were determined by assay of a composite of three 24-hour urine collections — the period of collection corresponding to the last three days of the dietary study. Creatinine excretion [6] was determined on each 24-hour collection. A daily excretion of 0.95 gm or above of creatinine was considered indicative of complete urine collections.

Concurrent with the study of the two groups of pregnant women, some information on the dietary intake, urinary excretion of N^1-methylnicotinamide and response to a 50 mg test dose of nicotinamide was obtained on 12 non-pregnant women. These data are included here for the purpose of comparing the pattern of excretion of the metabolic products of nicotinic acid

[6] The authors were very kindly given access to the results of creatinine determinations which were being made in the laboratories of the Chicago Lying-in Hospital.

by pregant and non-pregnant women. The data on the dietary intake of nicotinic acid of the 12 women in group III were based upon calculated values of two week-day records of food intake estimated in grams. Twenty-four-hour urine collections were made the second day of the dietary study. The response to a 50 mg test dose of nicotinamide on the third day was determined for this group of subjects according to the procedure described above for group I.

The N^1-methylnicotinamide in urine was determined by the acetone-fluorometric method of Huff and Perlzweig ('47). Digests of the diet and urine composites, made with 2 *N* H_2SO_4, were assayed for nicotinic acid by the chemical method (cyanogen bromide-amine reaction) as modified by one of the present authors (Frazier, '47). Aliquots of the digest of diet composites were extracted with chloroform before assay, since fat was found to interfere with the chemical assay. The acid digest of the urine included free nicotinic acid, hydrolyzed nicotinamide and any nicotinuric acid present.

RESULTS

The relation of the dietary intake of nicotinic acid to the urinary excretion of some of its metabolic products

Group I. The daily intake of nicotinic acid and the urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, as well as the percentage recovery of a 50 mg test dose of nicotinamide, for the 7 primigravidae during each of the 5 7-day study periods are presented in table 1. There was considerable variation from month to month in the intake of nicotinic acid by individual subjects. The average daily intake for the 5 subjects on unsupplemented dietaries ranged from 13.8 to 18.3 mg. The average daily intake for all subjects was 19.7 mg.

The average daily excretion of the acid-hydrolyzable metabolites of nicotinic acid was 1.1 mg. The level of excretion of

TABLE 1

The nicotinic acid intake, urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, and per cent recovery of a 50 mg test dose of nicotinamide by 7 primigravidae from the 5th through the 9th months

SUBJECT	STUDY PERIOD	INTAKE NICOTINIC ACID	EXCRETION, EXPRESSED AS NICOTINIC ACID: Acid hydro-lyzable	N^1-methyl-nicotin-amide	Total: Amount	Total: Fraction of intake	RECOVERY OF TEST DOSE
	mo.	mg	mg	mg	mg	%	%
1	5	16.6	1.7	7.3	9.0	54	15.6
	6	16.9	1.4	6.8	8.2	48	15.1
	7	18.7	1.0	8.8	9.8	52	21.0
	8	15.5	1.4	13.0	14.4	93	20.0
	9	15.0	1.4	13.1	14.5	97	23.3
	Average	16.6	1.4	9.8	11.2	67	19.1
2	5	16.2	1.0	9.1	10.1	62	15.8
	6	16.6	0.5	9.0	9.5	51	14.1
	7	12.2	0.7	12.4	13.1	107	18.0
	8	9.8	0.8	8.1	8.9	91	28.3
	9	14.4	0.9	17.7	18.6	128	24.4
	Average	13.8	0.7	11.3	12.0	87	20.1
3	5	22.9	1.0	10.3	11.3	49	19.0
	6	16.4	0.7	9.9	10.6	64	8.0
	7	20.9	1.3	14.0	15.3	73	19.8
	8	17.2	1.1	15.1	16.2	94	10.1
	9	14.3	0.9	13.9	14.8	102	13.1
	Average	18.3	1.0	12.6	13.6	74	14.0
4	5	12.9	0.8	10.8	11.6	90	16.2
	6	17.2	1.0	10.7	11.7	68	15.1
	7	21.6	0.7	14.0	14.7	68	19.5
	8	18.3	1.1	16.9	18.0	98	22.9
	9	16.6	1.7	15.8	17.5	106	13.8
	Average	17.3	1.0	13.6	14.6	83	17.1
5	5	18.3	1.3	10.3	11.6	63	12.0
	6	20.6	1.2	15.1	16.3	80	16.7
	7	11.8	1.3	13.5	14.8	125	20.3
	8	12.6	0.9	13.5	14.4	114	19.5
	9	20.2	1.6	10.6	12.2	60	17.2
	Average	16.7	1.3	12.0	13.5	80	17.1
6	5	18.6	0.7	10.5	11.2	60	23.8
Supplement	6	11.8	1.3	9.8	11.1	94	8.4
11.2 mg	7	24.6	1.1	14.5	15.6	63	23.8
per day 7,	8	28.4	0.9	14.8	15.7	55	16.7
8, 9 mos.	9	24.9	0.9	15.6	16.5	66	17.5
	Average	21.7	1.0	13.0	14.0	64	18.0
7	5	31.5	2.2	23.7	25.9	82	23.2
Supplement	6	37.1	1.0	22.7	23.7	64	25.9
20.2 mg per	7	33.6	1.2	22.2	23.4	69	24.5
day all	8	34.0	2.1	23.1	25.2	74	32.2
periods	9	32.8	1.3	14.8	16.1	49	...
	Average	33.8	1.6	21.3	22.9	68	26.5
All	Average	19.7	1.1	13.8	14.9	75	18.8

TABLE 2

A summary of the average daily intake of nicotinic acid and urinary excretion of some of its metabolites by primigravidae

MONTH OF PREGNANCY	NUMBER OF SUBJECTS	INTAKE NICOTINIC ACID	EXCRETION				FRACTION OF INTAKE EXCRETED		
			Acid hydrolyzable	N^1-methylnicotinamide	Nicotinic acid equiv.	Total	Nicotinic acid	N^1-methylnicotinamide	Total
		mg	mg	mg	mg	mg	%	%	%
A. Subjects on both unsupplemented and supplemented dietary intakes									
5	7	19.6	1.2	13.0	11.7	12.9	6	60	66
6	7	19.5	1.0	13.3	12.0	13.0	5	62	67
7	7	20.5	1.0	15.8	14.2	15.2	5	69	74
8	7	19.4	1.2	16.6	14.9	16.1	6	77	83
9	7	19.7	1.2	16.1	14.5	15.7	6	74	80
B. Subjects on unsupplemented dietary intakes									
5	6	17.6	1.1	10.8	9.7	10.7	6	57	63
6	6	16.6	1.0	10.2	10.2	11.2	6	61	67
7	5	17.0	1.0	14.1	12.5	13.5	6	72	80
8	5	14.7	1.1	14.8	13.3	14.4	7	91	98
9	5	16.1	1.3	15.8	14.2	15.5	8	90	98

A statistical covariance analysis [7] of these data showed that with the elimination of the influence of intake the variation in excretion of the total metabolic products studied was not significant. However, this analysis *did* show significant differences among the patterns of excretion of individual subjects. Accordingly, a covariance analysis of total excretion (Y) on intake (X) for the 5 subjects on unsupplemented intakes with similar patterns of urinary excretion (table 2, part B) showed no significant differences among the subjects.

TABLE 3

The relation of dietary intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide by 12 pregnant women at Chicago Lying-in Hospital clinics

TRIMESTER OF PREGNANCY	NO OF SUBJECTS	AVERAGE DAILY INTAKE NICOTINIC ACID	AVERAGE DAILY EXCRETION	
			N^1-methyl nicotinamide (nicotinic acid equiv)	Fraction of intake
		mg	mg	%
1	5	14.8	11.2	75
2	19	12.4	10.9	80
3	18	11.1	13.1	118
	42	12.1	11.9	98

A test of significance between the adjusted means for each of the 5 months showed, however, a significant difference in total excretion during the last trimester at the 5% probability level.

Confidence limits for population months of pregnancy indicated no significant difference in total excretion by the subjects between the two months studied in the second trimester, or between the three months in the third trimester.

Thus, there is statistical evidence that a real difference exists in the levels of excretion of N^1-methylnicotinamide by primigravidae on unsupplemented dietary intakes of nicotinic acid with progression of pregnancy. Moreover, the statistical analysis verifies the observation that the pattern of excretion

[7] The authors are indebted to Dr. T. A. Bancroft, Director of the Statistics Laboratory, Alabama Polytechnic Institute, for statistical analysis of these data.

of the two subjects on the supplemented intakes was significantly different from that of the 5 subjects on unsupplemented dietary intakes.

Group II. Table 3 presents the data on the average daily intake of nicotinic acid, excretion of N¹-methylnicotinamide and percentage of intake of this metabolite excreted, for 42 clinic subjects grouped according to trimester of pregnancy.

There was considerable variation in dietary levels of nicotinic acid within this group. The total average calculated intake was 12.1 mg, which represented a range of from 6.4 to 24.5 mg per day. In table 4 the subjects have been grouped

TABLE 4

The relation of dietary intake of nicotinic acid to urinary excretion of N¹-methylnicotinamide by 42 patients of Chicago Lying-in Hospital clinics

NUMBER OF SUBJECTS	INTAKE		EXCRETION		FRACTION OF INTAKE EXCRETED
	Average	Range	N¹-methyl-nicotinamide	Nicotinic acid equivalents	
	mg	*mg*	*mg*	*mg*	%
4	6.4	4.6–7.4	12.4	11.2	175
11	8.7	7.5–9.9	12.1	10.9	125
12	11.3	10.0–12.4	12.7	11.4	100
6	13.4	12.5–14.9	13.4	12.1	90
9	18.4	15.0–24.5	14.1	12.7	62
42	12.1		13.1	11.9	98

according to range of intake as follows: Those who received 4.6–7.4; 7.5–9.9; 10.0–12.4; 12.5–14.9; and 15.0–24.5 mg per day. On intakes within these ranges the average amounts of N¹-methylnicotinamide excreted (expressed as nicotinic acid) were 11.2, 10.9, 11.4, 12.1 and 12.7 mg, respectively. These data indicate no apparent relation between intake of nicotinic acid and excretion of N¹-methylnicotinamide; rather, they indicate a more or less constant level of excretion of the methylated derivative regardless of intake (fig. 2).

The data for this group of subjects confirm the observations on the subjects in group I in two important respects. First,

the 9 subjects in group II with an average intake of 18.4 mg excreted an average of 14.1 mg of N^1-methylnicotinamide per day (table 4) while the 7 subjects in group I on a comparable intake of 19.7 mg excreted an average of 13.8 mg of this metabolite (table 1). Second, there was also an increase in level of excretion of N^1-methylnicotinamide by this group with increased duration of pregnancy, as in the case of the 5 primigravidae in group I on unsupplemented intakes. The average percentages of intake excreted were 75, 80, and 118 for the first, second and third trimester, respectively (*table 3*). *Fourteen* of the clinic subjects were primigravidae, 7 in each of the

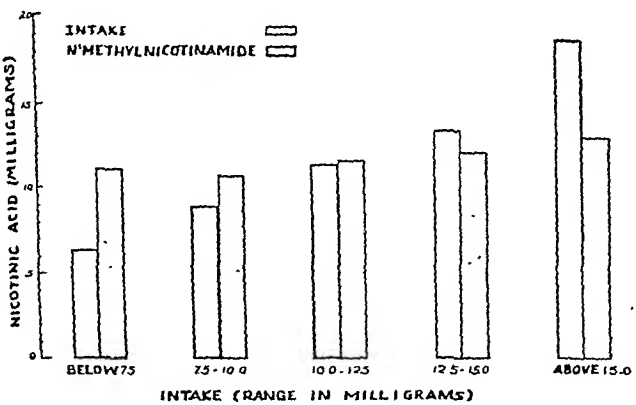

Fig. 2 The relation of dietary intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide (expressed as nicotinic acid) of pregnant women.

second and third trimesters. This group is comparable, therefore, to the 5 subjects in group I with respect to gravida and trimester of pregnancy; it differs, however, in precision of measurement, since the excretion values on the 5 subjects in group I were means of 5 study periods of 7 days each, while the 14 observations on subjects in group II represent single study periods of three days each. A statistical covariance analysis of excretion (Y) on intake (X) showed no significant difference in performance between the two groups when a test of significance of the adjusted excretion means was made.

Group III. The data on the nicotinic acid intake, excretion of N¹-methylnicotinamide, and the percentage recovery of a 50 mg test dose of nicotinamide over a 4-hour period for the 12 non-pregnant women are shown in table 5.

There was a considerable range in intake, from 8.6 to 15.9 mg, with an average for all subjects of 12.7 mg per day.

TABLE 5

The dietary intake of nicotinic acid (calculated) and the urinary excretion of N¹-methylnicotinamide by non-pregnant women

SUBJECT	INTAKE PER DAY	EXCRETION			FRACTION OF 50 MG NICOTINAMIDE
		N¹-methyl nicotinamide	Nicotinic acid equivalent	Fraction of intake	
	mg	*mg*	*mg*	%	%
A	14.1	4.0	3.6	25.5	9.1
B	14.5	3.4	3.1	21.3	13.8 [1]
C	13.0	3.3	2.9	22.3	3.0
D	13.2	5.1	4.6	42.4	4.6
E	11.1	4.9	4.4	39.6	9.0
F	10.2	9.7	8.7	85.3	7.1
G	8.6	5.3	4.8	55.8	11.5
H	15.9	7.8	7.0	44.0	6.7
I	15.7	5.2	4.7	30.0	7.0
J	10.9	8.7	7.8	72.2	11.2
K	13.0	7.2	6.5	50.0	10.4
L	12.4	7.4	6.7	54.0	8.9
Average	12.7	6.0	5.4	42.5	8.4 [2]

[1] Seventy-five milligram test dose.
[2] Exclusive of subject B.

Again, there was variation among subjects in the amount of N¹-methylnicotinamide excreted irrespective of intake. For instance, subject B on an intake of 14.5 mg of nicotinic acid excreted 3.1 mg of N¹-methylnicotinamide (expressed as nicotinic acid) or 21.3% of the intake. Subject F, on the other hand, excreted 8.7 mg, or 85.3% of an intake of 10.2 mg.

The level of excretion of the methylated derivative, however, was much lower than was found in the case of the preg-

nant women. The average daily excretion of this compound was 6.0 mg, or 5.4 mg nicotinic acid equivalents. The average percentage of intake excreted by this group of subjects was 42.5.

Response to a test dose of nicotinamide

The data on the percentage recovery of the 50 mg test dose of nicotinamide over the 4-hour period for the 7 primigravidae in group I, and for the 12 non-pregnant women in group III, are shown in tables 1 and 5, respectively. There was some variation in response to the test dose among subjects within

TABLE 6

The relation of dietary intake of nicotinic acid to the average percentage return of a 50 mg test dose of nicotinamide by primigravidae

SUBJECT BY NUMBER	INTAKE OF NICOTINIC ACID	RETURN OF 50 MG OF NICOTINAMIDE
	mg	%
2	13.8	20.1
1	16.6	19.1
5	16.7	17.1
4	17 3	17.1
3	18.3	14.0

the groups, as well as among study periods of individual subjects in group I.

In general there was a slight tendency toward an increase in percentage return by the pregnant women from the 5th through the 9th month. Again the performance of subject 7 differed from that of the subjects on the lower unsupplemented dietary intakes. Although the data for this subject are incomplete due to the fact that she delivered before the completion of the last study period, the percentage recovery of the test dose was consistently higher than that of the other subjects and was fairly constant.

Both groups I and III showed a small but consistent decrease in the percentage return of the 50 mg of nicotinamide

with increased levels of intake. The relation of dietary intake to average percentage return of the test dose is shown in table 6, which lists the 5 subjects in group I on unsupplemented dietary intakes in ascending order according to average intake of nicotinic acid. Although the dietary data on the nonpregnant women were less precise, a similar trend in decreased percentage return of the test dose with increased intake of nicotinic acid was observed among these subjects (table 5).

DISCUSSION

The function of the coenzymes in carbohydrate metabolism suggests a direct relationship between the production of energy and the utilization of nicotinic acid by the body. Sandiford, Wheeler and Boothby ('30) found that total heat production during pregnancy increased gradually from the end of the third month. During later pregnancy the rate increased more rapidly, and reached its highest point just before delivery. The fact that the basal metabolic rate immediately following delivery was comparable with the average rate during the first lunar month, and that the increased rate during late pregnancy could be accounted for by added metabolism of fetus, placenta, and accessory structures, led these investigators to conclude that there was probably no change in actual rate or intensity of heat production per unit mass of maternal tissue. This seems to suggest that the observed increase in level of excretion of N^1-methylnicotinamide during the last trimester of pregnancy might be attributed to an increase in the requirement of the maternal organism for coenzymes I and II paralleling the increase in total body metabolism. The conclusions of Sandiford et al. further suggest a participation of the fetal liver in the total methylation of the pyridine compound during prenatal life.

This reasoning is based upon the assumption that the methylated derivative is a true metabolite of nicotinic acid, i.e., that it is an end product of intermediary metabolism and that an increased excretion of N^1-methylnicotinamide is indicative of an increased rate of turnover of the coenzymes.

The increase in percentage return of a test dose of nicotinamide by the subjects in groups I and III with decreased levels of dietary intake supports this hypothesis.

Thus it is conceivable that with the increased demands of the maternal organism more of the vitamin is mobilized to meet body needs, which results in an increased excretion of its metabolites. This interpretation of the pattern of urinary excretion of the methylated derivative of this vitamin is directly opposite to the usual interpretation of an increased excretion of the water soluble vitamins following test doses; namely, that the higher the quantity of the test dose returned the lower the quantity of the test dose utilized. Clarification of this problem must await additional information on the level of excretion of the metabolite of nicotinamide, N^1-methyl-6-pyridone-3-carboxylamide, first described by Knox and Grossman ('47), as well as on the interrelation of tryptophan and nicotinic acid on known levels of intake.

An examination of the food intakes of the subjects in group I revealed that the level of protein consumed by each subject was within normal limits as to quantity; was derived largely from milk and milk products, meat, eggs, and cereals; and did not *significantly* change either in character or amount throughout the 5-month period of study. Actually there was a slight decrease in daily intake of protein (8–10 gm) in the 9th month as compared to the 5th month in 6 of the 7 subjects in this group. This slight decrease in protein intake may have been a reflection of a seasonal difference in the types of foods prepared and selected by the subjects in their winter and summer diets, since most of the subjects delivered in the summer. Although no quantitative data on tryptophan intakes were determined from these diets, it would appear unlikely that tryptophan intakes increased as pregnancy progressed.

The constant levels of excretion of N^1-methylnicotinamide by subjects 6 and 7, and the higher excretion by subject 7, on supplemented intakes of nicotinamide indicate that, with the ingestion of a constant relatively high amount of the amide over a period of time, there is a turnover of coenzymes suf-

ficient to meet, or even in excess of, the increased demand during later pregnancy. The uniform response of subject 7 to the test dose also reflects this concept. The failure of these two subjects to show an increased excretion of N^1-methylnicotinamide during the last trimester suggests that an intake of 20 mg or above of nicotinic acid is in excess of the daily requirements during pregnancy.

The average 24-hour excretion of 5.4 mg equivalents of nicotinic acid as the methylated derivative by the normal controls in group III approximates that reported by other investigators for healthy adult women on average diets. On intakes of approximately 10 mg Ellinger and Benesch ('45) report an average daily excretion of 3.8 mg of nicotinic acid equivalents by 5 adult women, as measured by a modification of the method described by Najjar ('44). Employing a modification of this method, Coryell et al. ('47) found an average excretion of 3.4 mg equivalents of N^1-methylnicotinamide (corrected for the 19% error) by 5 women during the lactation period of from 68 to 306 days *post partum*. An examination of these data shows a slight but consistent increase in excretion of N^1-methylnicotinamide during late lactation, an approach to the values obtained for normal adult women. Perlzweig and associates ('47) found an average excretion of 5.9 mg of nicotinic acid equivalents by three adult women on "good average diets." These data were obtained by the method of assay employed in the present study (Huff and Perlzweig, '47) and agree with the average excretion for the normal controls in group III of 5.4 mg.

The constant level of excretion of the acid-hydrolyzable nicotinic acid of from 5 to 8% of the intake (table 2) is consistent with that observed by other investigators (Sarett, Huff and Perlzweig, '42; Johnson, Hamilton and Mitchell, '45; Oldham, Davis and Roberts, '46), all of whom found similar levels of excretion of the acid-hydrolyzable fractions regardless of the levels of intake. Such a level of excretion may represent the renal threshold for this group of compounds — namely, nicotinic acid, nicotinamide, and nicotinuric acid.

SUMMARY

1. The dietary intake of nicotinic acid, the urinary excretion of the acid-hydrolyzable fraction and N^1-methylnicotinamide, and the response to a 50 mg test dose of nicotinamide, were studied on 7 primigravidae during each of the last 5 months of pregnancy. In addition, a study was made of the dietary intake of nicotinic acid and the urinary excretion of N^1-methylnicotinamide by 42 clinic subjects. The estimated intake of nicotinic acid, the urinary excretion of N^1-methylnicotinamide and the response to a 50 mg test dose of nicotinamide were observed on 12 non-pregnant women.

2. There was a constant level of excretion of the acid-hydrolyzable fraction regardless of intake.

3. There was a statistically significant increase in the excretion of N^1-methylnicotinamide by the 5 primigravidae in group I on unsupplemented diets during the last trimester of pregnancy. This observation was confirmed by the data on the 14 primigravidae in group II.

4. The significance is discussed of the relation of the intake of nicotinic acid to the urinary excretion of N^1-methylnicotinamide and the percentage return of a 50 mg test dose of nicotinamide.

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THE DETERMINATION OF EARLY THIAMINE-DEFICIENT STATES BY ESTIMATION OF BLOOD LACTIC AND PYRUVIC ACIDS AFTER GLUCOSE ADMINISTRATION AND EXERCISE [1]

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FIVE FIGURES

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It is important to develop a method which will evaluate metabolic insufficiency prior to the development of the more obvious clinical signs of thiamine deficiency. Low levels of thiamine in blood and urine give valuable information about the recent dietary history of the patient, but a method which identifies the disturbance of cell metabolism resulting from these dietary inadequacies facilitates more specific interpretations.

Friedemann and Barborka ('41) have shown that there is a definite ratio between levels of lactate and pyruvate in the blood. Stotz and Bessey ('42), working with pigeons, advocated the use of blood lactate-pyruvate ratios to distinguish true abnormality of pyruvate metabolism from the fluctuations due to exercise, food consumption and anoxia. As a result of the emphasis placed upon increases in pyruvic acid associated with thiamine deficiency there have been many clinical attempts to use the basal level of pyruvic acid in the blood as an indica-

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tion of the degree of thiamine deficiency of patients. On the whole there has been little agreement between values of blood pyruvic acid and the degree of early thiamine deficiency states (Keys, Henschel, Taylor, Mickelsen and Brozek, '45; Berryman et al., '47). This discrepancy may be due either to the variability of the basal level of the pyruvic acid or to the lack of agreement regarding the sub-clinical signs of thiamine deficiency, or to both reasons.

Bueding, Stein and Wortis ('40) showed that the ingestion of glucose caused a temporary but significant elevation of blood pyruvic acid, which was abnormally prolonged and elevated in thiamine deficiency. Williams, Mason, Smith and Wilder ('40) made similar observations correlating the levels of the lactic and pyruvic acids after glucose administration during experimentally produced thiamine deficiencies. The opportunities provided by a long-term controlled study in thiamine deficiency (Horwitt, Liebert, Kreisler and Wittman, '48) made it possible to test published procedures for evaluating thiamine deficiency and to develop a method which determines the combined effects of glucose ingestion and mild exercise on the blood lactic and pyruvic acids.

METHODS

Glucose was estimated by the procedure described by Hoffman ('37). The *pyruvic acid* and *lactic acid* methods used were those reported by Friedemann and Haugen ('43) and Barker and Summerson ('41), respectively. Modifications in these methods designed to insure reproducibilty of results and special precautions employed in the handling of blood have been described elsewhere (Horwitt et al., '48). *Oral glucose* was administered immediately after withdrawal of the fasting basal blood sample (9 ml 20% glucose per kilogram). *The mild exercise test*, which was applied 60 minutes after glucose ingestion, consisted of walking down and up, and then down and up again, a flight of 21 steps, each step being 19 cm high. The rate of descent and climb was controlled so that the exercise was completed in 60 seconds. The lactic and pyruvic acids reach

their peak about 5 minutes after exercise; therefore the blood drawn at that time is called the 66 minute sample.

The details of the *human thiamine depletion study* and the elaborate dietary and analytical precautions taken to insure accuracy of food intake have been described elsewhere (Horwitt, Liebert, Kreisler and Wittman, '48). In summary, 36 male patients were selected from the State Hospital population and divided into three groups, A, B and C. Each person in the A group received a daily diet containing approximately 2,200 calories, which was apparently adequate in all the essentials except thiamine, of which approximately 400 µg were present, and riboflavin, of which the diet contained about 900 µg. Those in the B group received the same diet during the first two years of the experiment except that a daily supplement of yeast extract was given, containing approximately 6 mg of thiamine and 1.3 mg of riboflavin. The members of the C group received the regular hospital diet, which they ate ad libitum. Dietary checks of their food consumption indicated that they ingested approximately 1 mg of thiamine per day.

The clinical effect of the restriction of thiamine to 400 µg per day in the A group was at no time more than minimum. Therefore, after 25 months of this regimen, it was decided to feed the B group a diet containing approximately half as much thiamine as the A group were receiving. Accordingly the supplementation of the B group was stopped and the subjects of this group were placed on a diet which provided approximately 200 µg of thiamine and 800 µg of riboflavin daily.

During the more than three years in which these subjects were observed, studies were made on the blood glucose, lactic and pyruvic acid levels basally, after glucose administration and after glucose followed by exercise. The results were correlated with the diets fed and with the clinical signs of thiamine deficiency which appeared.

RESULTS

The *basal blood levels of lactic and pyruvic acid* did not correlate well with the states of thiamine deprivation. The 5 young

men in the B group, after two months on 200 µg of thiamine daily, had levels of 1.05, 1.05, 1.07, 0.82, and 1.05 mg % for pyruvic acid, respectively, which were similar to those they had shown for the previous two years. After 4 months on this diet, when most of them showed load-test evidence of abnormality, they had levels of 0.96, 1.24, 1.22, 1.06, and 1.39 mg %, respectively. The old men in the B group had control basal levels of 0.86, 0.68, 0.96, 0.66 and 0.98 mg %, respectively, and after two months on the restricted diet, levels of 0.80, 1.12, 0.76, 1.29, 1.03 and 0.97. Only after 4 months, when the load-test results were all abnormally high, were the basal pyruvate levels somewhat higher, being 1.18, 1.50, 1.36, 1.10 and 1.20 mg % (1 lost). While there was this trend toward higher basal levels with continued dietary restriction, the amount of the increase was not significant in most cases. Experience with several hundred individuals on apparently normal diets has indicated that the basal pyruvic acid levels show large variations that are not consistent with levels obtained at 60, 66 and 120 minutes after glucose ingestion. Though it is proper to suspect pathology whenever a basal pyruvic acid level exceeds 1.20 mg %, such levels are not uncommon, even in the absence of any apparent metabolic disorders. It is significant, however, that the individual (BO2) who showed the most advanced neurological pathology had the two highest basal levels of pyruvic acid, 1.50 and 1.98 mg %, the latter taken at the height of his clinical pathology.

The basal lactic acid data showed an approximate 10 to 1 correlation with pyruvic acid. Thus, on the day that the basal pyruvic acid levels for the old subjects of the B group were 1.18, 1.50, 1.36, 1.10 and 1.20 mg %, the lactic acid values were 11.5, 15.6, 11.9, 11.1 and 11.3 mg %, respectively.

Glucose as a "metabolic load"

The changes in blood levels of lactic and pyruvic acid after glucose administration were determined bimonthly. Data obtained at 60 and 90 minutes after glucose administration have

been recorded previously (Horwitt et al., '48). These data indicated that though the average results with the depleted subjects were somewhat higher than those with subjects on an adequate thiamine intake, the individual figures overlapped. The averages, while significant for a group, were not indicative of a test sufficiently sensitive to permit the detection of early abnormality in the individual.

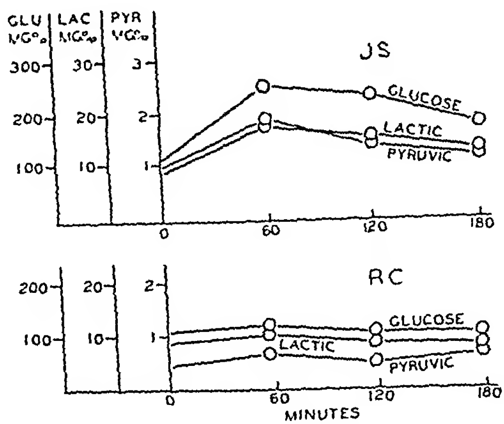

Fig. 1 Comparison of relative changes in lactic and pyruvic acid with high and low glucose tolerance curves.

Glucose plus exercise as a "metabolic load"

Mild exercise alone as a "metabolic load" showed promise of distinguishing deficient from non-deficient subjects. This criterion was abandoned, however, because the exercise test used produced only a small rise in lactic and pyruvic acids and the unreliable base line made such a rise difficult to interpret. Severe exercise tests could not be used since they provide data which are too dependent upon cooperation, training and cardiovascular efficiency. The near success of the mild exercise test prompted the next step, namely, to combine glucose ingestion and mild exercise to form a double "metabolic load." It was reasoned that this would provide a greater strain on the mechanisms of carbohydrate metabolism and also make it possible

to utilize the more accurate 60 minute base line. The results of this glucose-exercise test (see Horwitt et al., '48) showed a more marked difference among the experimental groups than appeared with glucose alone, but individual variations gave evidence that the glucose levels should be considered in order to avoid misinterpretations of the data.

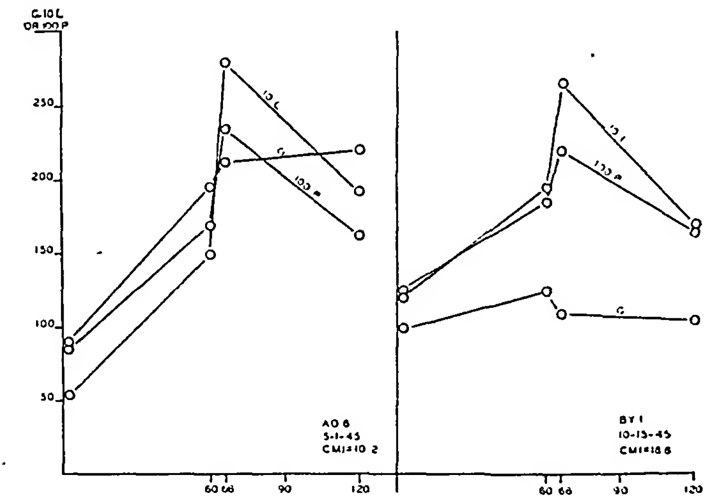

Fig. 2 Comparison of glucose, lactate and pyruvate results obtained from subject AO6, who showed no positive signs of clinical thiamine deficiency at the time of test, and subject BY1 when the latter was showing definite signs of thiamine depletion. Lactic and pyruvic acid results are multiplied by 10 and 100, respectively, to bring them to the same scale as glucose.

Correlation of blood glucose with blood lactate and pyruvate

The importance of this step is more apparent if the lactic and pyruvic acid levels of patients with hypoglycemic and hypergylcemic glucose tolerance curves are compared. Figure 1 presents an example of observations on two such individuals, both of whom were receiving adequate amounts of thiamine. The subject, J. S., with a curve illustrative of alimentary hyperglycemia, had high levels of lactic and pyruvic acid by usually accepted standards, but he also had glucose levels which were high. Conversely, subject R. C., with a flat glucose

tolerance curve, had correspondingly low lactic and pyruvic acid levels. Figure 2 presents data from two subjects on thiamine-deficient diets. One, BY1, was consuming the diet containing 200 μg of thiamine daily; the other, AO6, the diet with 400 μg. The former showed definite signs of clinical thiamine deficiency; the latter, although having equally high

TABLE 1

Relationship of glucose and lactic and pyruvic acid before and after exercise[1]

TIME	BASAL			60 MINUTES			66 MINUTES			120 MINUTES		
Subject	Gluc.	Lact.	Pyr.	Gluc.	Lact.	Pyr	Gluc.	Lact.	Pyr.	Gluc.	Lact.	Pyr.
AY3	118	9.0	1.14	231	20.5	2.12	188	30.5	2.54	114	19.3	1.76
AY4	110	11.5	0.99	200	25.6	2.32	168	31.1	2.28	154	20.6	1.08
BO5	107	8.4	1.08	233	16.5	1.38	199	22.3	1.33	196	15.4	1.23
BY5	104	9.0	1.08	170	16.6	1.52	135	20.2	1.30	125	12.9	1.10
T. M.	121	6.9	0.69	218	20.1	2.00	176	19.8	1.53	137	13.8	1.50
J. S.	106	9.7	1.23	195	15.4	1.56	173	18.2	1.57	146	11.5	1.12
E. D.	120	8.6	1.00	206	12.5	1.05	175	22.0	1.43	158	9.4	0.72
F. D.	105	6.6	0.85	197	13.0	1.04	155	17.5	1.50	125	12.5	1.14

[1] One minute of stair climbing was instituted 60 minutes after the ingestion of glucose. The 66 minute samples were obtained 5 minutes after the completion of this exercise. These data are selected to show how the ratio is affected in situations where there is an unusual decrease of blood glucose following exercise, sometimes necessitating a decrease in pyruvic acid. This demonstrates the tendency toward maintenance of the lactate to pyruvate post-exercise ratio of approximately 15 to 1.

levels of lactic and pyruvic acid, showed no clinical signs of deficiency. However, when the levels of glucose were taken into consideration, it became apparent that AO6 was functioning at a higher blood sugar level and therefore was under a greater "metabolic load" at the time of the test.

Comparison of lactic and pyruvic acid levels of different individuals can only be made if they have similar levels of glucose. In other words, a high lactate or pyruvate figure should not be considered pathological unless it has been compared with the blood glucose level obtained at the same time,

due to the fact that the latter level is indicative of a greater or lesser load being applied to the enzyme systems involved.

Individuals in a satisfactory state of alimentation, when given the glucose-plus-exercise test, had levels of lactic acid 5 minutes after the exercise which were approximately one-tenth their levels of glucose, and levels of pyruvic acid which were approximately one-fifteenth their levels of lactic acid. When the increase of lactate after applied exercise is minimum, the increase of pyruvate is small or absent, thereby maintaining the approximate ratio of 1 to 15. This hypothesis was tested in those cases in which the glucose level showed a greater than average decrease after exercise. The data given in table 1 were chosen from all the experiments conducted during the past 4 years and represent atypical decreases in blood glucose obtained after exercise. By comparing the lactic and pyruvic figures at 60 minutes with those at 66 minutes (table 1), one may note how often the pyruvic acid does not increase following exercise, thus maintaining the ratio of approximately 1 to 15. Usually the glucose does not decrease appreciably after exercise and there is a greater increase in lactic acid which is in turn accompanied by a more definite rise in pyruvic acid, thus maintaining this approximate ratio.

The index of carbohydrate metabolism

Inasmuch as differences due to variations in glucose tolerance appeared quite frequently, a simple arithmetical expression was developed to relate the values of lactic and pyruvic acid to those of glucose. The formula is based on analysis of tests on several hundred different subjects. In each instance the blood levels for glucose, lactic acid and pyruvic acid were those obtained 5 minutes after the completion of the mild stair-climbing test applied 60 minutes after the oral administration of 1.8 gm of glucose per kilogram of body weight. The formula is

$$\mathrm{CMI} = \frac{L - \frac{G}{10} + 15\,P - \frac{G}{10}}{2},$$

where CMI = the index of carbohydrate metabolism, and where G, L, and P, are the levels of glucose, lactic acid and pyruvic acid in mg %, respectively. The possibility of simplifying the formula by using only one of the two metabolites was tempting; i.e., $CMI = L - \frac{G}{10}$ or $CMI = 15\,P - \frac{G}{10}$. Analysis of the data showed that such a simplification was adequate in most cases, and certainly in all cases with a very abnormal index. However, there were many cases where $L - \frac{G}{10}$ did not

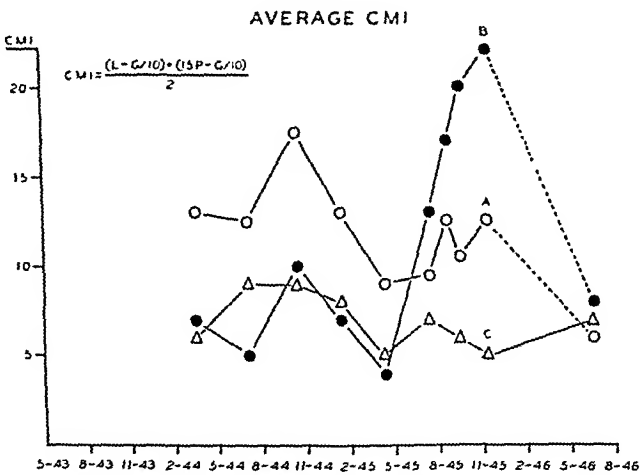

Fig. 3 Indices of carbohydrate metabolism (CMI) for the three dietary groups from March, 1944 to August, 1946. Group A received 400 μg thiamine from May, 1943 to December, 1945. Group B received the same diet plus yeast extract from May, 1943 to June, 1945, at which time their thiamine intake was decreased to 200 μg per day. Group C consumed the regular hospital diet ad libitum. The interrupted lines represent concluding periods of supplementation.

agree with $15\,P - \frac{G}{10}$, due to the fact that a state of equilibrium between lactic and pyruvic acids following exercise had not yet been attained when the blood sample was drawn. In such cases the average of the two gave a more reasonable figure, and one which might be considered more accurate statistically.

The results obtained by calculating the average CMI for groups A, B, and C are shown by graphs in figure 3. Since the glucose-exercise test was not devised until March, 1944, which was 9 months after the start of the dietary phase of the study,

these curves begin at that time. Note that the B group, which was yeast-supplemented until June, 1945, showed a marked increase in CMI when they were placed on 200 µg of thiamine per day, which was half as much as the A group continued to receive. Vitamin supplementation was begun for the indi-

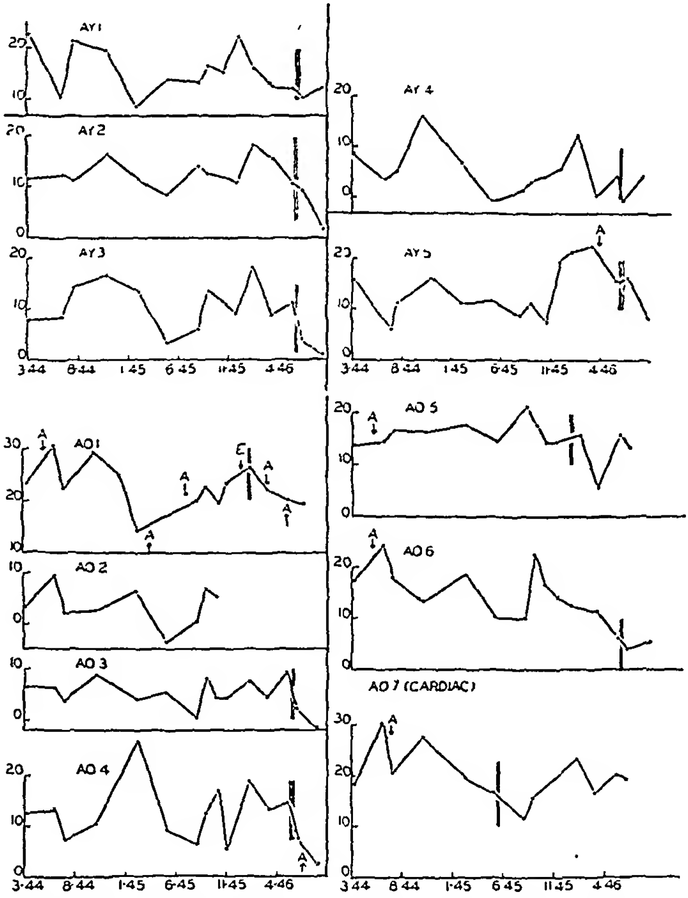

Fig. 4 Indices of carbohydrate metabolism for individual members of the A group. First such test (March, 1944) was not completed until 9 months after the start of the moderately restricted diet (400 µg per day). The multiple vertical lines represent times of supplementation. A and E with descending arrows indicate time at which there was an apparent change in the Achilles tendon reflex and some appearance of edema, respectively. The same symbols with ascending arrows indicate time at which these symptoms began to disappear. Such changes were not marked in this group.

viduals of both these groups after November, 1945. The curve for group C, in which the regular hospital diet was taken ad libitum, remained in a constant narrow range throughout the experiment.

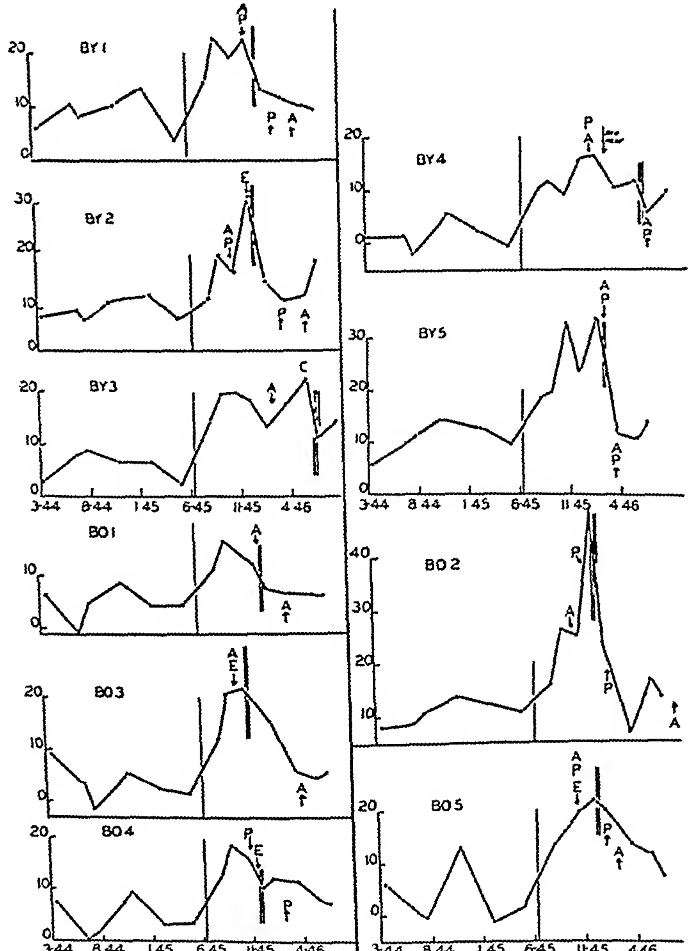

Fig. 5 Indices of carbohydrate metabolism for individual members of the B group. Their depletion period (200 μg per day) started in June, 1945. The multiple vertical lines represent times of re-supplementation. A, P, and E with descending arrows indicate first time at which definite signs of clinical deficiency as manifested by Achilles tendon reflex diminution, patellar reflex diminution, and edema, respectively, were noted. Such changes became very marked in this group. The ascending arrows indicate time when these symptoms began to disappear.

The advantage of the CMI over the levels of lactic and pyruvic acids as a measure of thiamine deficiency is demonstrated even better by the results obtained with individual subjects (figs. 4 and 5). There were numerous instances of non-depleted subjects in whom high lactic and pyruvic acid levels were associated with and dependent on simultaneously high levels of glucose. However, in no case did a subject in the B group reach a CMI of more than 15 during the prolonged period of adequate thiamine supply (prior to June, 1945), whereas every subject in this group went higher than 15 within 5 months after severe thiamine depletion and most of them did so within three months. This occurred in every case weeks before clinical symptoms of thiamine deficiency were noted.

The CMI of individuals in the A group (fig. 4) on the borderline thiamine deficiency diet (400 µg per day) fluctuated above and below the value 15, suggesting that these subjects were shifting in and out of a state of biochemical abnormality. The symbols A, P, and E with descending arrows (figs. 4 and 5) indicate the time at which the first clinical signs of thiamine deficiency appeared, as manifested by Achilles tendon reflex diminution, patellar reflex diminution and edema, respectively. The same symbols with ascending arrows indicate the time at which these symptoms disappeared after thiamine was restored by supplementing the diet. The single vertical line represents the time at which the B group was changed from a supplemented diet to the one containing 200 µg of thiamine per day. The multiple vertical lines indicate the time when a daily supplementation with at least 2 mg of thiamine was started. The CMI of all members of the B group promptly fell to below 15 following this renewal of the thiamine supply.

The CMI level of 15 was chosen as the approximate upper limit of normal because this figure was not surpassed by the subjects on an adequate diet and was invariably surpassed by those on a regimen grossly deficient in thiamine. The consistency with which the CMI levels of the B group rose from below 15 when the subjects of this group were placed upon the more depleted diet adds to the significance of the oscillations

observed in the CMI levels of the subjects of the A group on their less depleted diet. As previously mentioned, it is believed that these oscillations signify that the diet of the A group was barely adequate in thiamine and that the subjects of the group were in and out of actual thiamine deficiency. It was observed that the individuals in the A group voluntarily decreased their physical activity periodically during the course of the experiment, this decrease being related to the frequent return of the CMI to a level below 15. Similarly, some of the recovery from the high CMI levels found in the B group at the height of their clinical deficiency and before treatment with thiamine was started, was associated with bed rest. It has been postulated that this demonstrated a decreased need for thiamine when the subjects, as a result of bed rest, were functioning under a minimum "metabolic load."

The CMI of the individuals in the C group are not presented here but it should be mentioned that these subjects, who were on the regular hospital diet, did not reach any values over 15 during the course of the experiment. The average CMI for group C is shown in figure 3.

Test of CMI on population groups

A preliminary test of the procedure based on CMI was made on a group of mental patients in a ward which was known to contain poor eaters. Their CMI was measured before, during and after 8 months of daily supplementation with more than 3 mg of thiamine. These patients, though probably in poor nutritional states, had no incontrovertible signs of thiamine deficiency. Of 33 patients who were available for the 8-month period, 9 started with CMI of 15 or over. After the supplementation period, 7 of these 9 had marked decreases (4 units or more) to below 15, one changed from 30 to 20, and the 9th increased insignificantly from 15 to 16. The CMI of all 9 patients decreased markedly during the first month of supplementation. Of the 24 patients who started at levels below 15, 4 had decreases in CMI of more than 4 units and two had small increases. The remainder were practically unchanged.

A similar study was conducted, simultaneously, on a comparable group of mental patients who worked on the hospital farm and who voluntarily ate more food, probably because of their outdoor activity. These patients showed no significant changes in their CMI. All of these individuals had results below 15 and compared well with the subjects in the C group reported above.

DISCUSSION

To attempt to compare the results obtained in the present study with the reports of other investigators may not be warranted because of differences in the diets, subjects, and duration of the experiments. Nevertheless one might be inclined on the basis of the work now reported to lay greater emphasis on the importance of the results of blood lactic and pyruvic acid levels than would be indicated by the work of Keys, Henschel, Taylor, Mickelsen and Brozek ('45), who claimed that only slight differences were obtained in blood pyruvic acid levels after glucose ingestion. Berryman et al. ('47) reported that the resting and post-exercise pyruvic acid levels were high in some of their experimental subjects.

The fundamental shortcoming of the basal lactic and pyruvic acid levels as a criterion of thiamine deficiency was the large variation in individual figures, regardless of the state of nutrition, as compared with the consistency of figures obtained 60 minutes after glucose ingestion. Although there was a tendency for the basal figures to increase as the deficiency progressed, the significance of this change was not apparent during the early development of deficiency. It was true, however, that many of the resting pyruvate levels were increased after other signs of thiamine deficiency had appeared.

The importance cannot be over-emphasized of evaluating the severity of the "metabolic load" under which a subject is functioning at the time when the blood lactic and pyruvic acids are determined. A level of blood glucose of 200 mg% is a different "load" than a level of 125 mg%. Furthermore, the amount of exercise should be limited. Not only is it simpler

to control the amount of exercise when it is mild, since the factor of volition becomes less important, but avoidance of severe anoxia minimizes the marked variations in levels of lactic and pyruvic acid which are obtained among individuals given strenuous exercise tests. An individual with a less efficient cardiovascular system will have different blood lactic and pyruvic acid levels after severe exercise than a trained athlete after the same exercise, but neither need be thiamine-deficient. Given a smaller "metabolic load" the physically trained and healthy untrained subject tend to give results which approach each other.

Our inclination to ascribe to thiamine the changes in blood lactate and pyruvic values in these experiments is reinforced by other recent observations. A year's subsistence of a group of subjects on a diet containing adequate thiamine but less than 600 µg of riboflavin per day did not produce any significant changes in blood lactic and pyruvic acids, even though characteristic lesions of the skin developed.

Thiamine deficiency is, of course, not the only metabolic situation which will elevate the blood levels of lactic and pyruvic acids. Cardiovascular insufficiencies, liver disorders, infections, thyrotoxicosis, and emotional excitement may all bring about increases. However, all of these conditions can be ruled out by a competent examiner. Data on the effects of disorders of metabolism will be presented in subsequent papers.

SUMMARY AND CONCLUSIONS

1. Thirty-six human subjects receiving diets of known low content of thiamine were studied from the standpoint of the relationship of the levels of lactic and pyruvic acid of the blood to the degree of thiamine deficiency.

2. The normal relationship between blood glucose levels and those of lactic and pyruvic acid was investigated, and the necessity demonstrated for considering this relationship in evaluating levels of blood lactate and pyruvate.

3. In the fasting state and in the absence of exercise there was no consistent rise in the levels of lactic and pyruvic acid

in mild degrees of thiamine deficiency, and it would seem that the organism at rest can maintain the normal equilibrium levels of these metabolites during mild thiamine deprivation. The basal levels were elevated in some cases of more severe thiamine deficiency but, even then, only after pronounced clinical signs of pathology were apparent.

4. The elevation of the lactate and pyruvate values of the blood which followed a combined "metabolic load" composed of ingestion of glucose and mild exercise was found to give more useful data than were obtained either after glucose ingestion alone or after exercise alone.

5. Under the conditions obtaining in the experiments here reported, the blood levels of lactic and pyruvic acid observed under this double "metabolic load" of glucose ingestion and exercise proved to be a means of diagnosing early and mild degrees of thiamine deficiency when these levels were correlated with the glucose level of the blood. The correlation was effected satisfactorily by the formula:

$$\mathrm{CMI} = \frac{L - \frac{G}{10} + 15\,P - \frac{G}{10}}{2},$$

where CMI stands for the index of carbohydrate metabolism and where G, L and P are levels of glucose, lactic acid, and pyruvic acid, respectively, in milligrams for each 100 ml of blood.

6. The usefulness of the determination for CMI was demonstrated in subjects on controlled thiamine-deficient diets of different severity. The use of the method to pick out thiamine-deficient individuals from population groups was also illustrated.

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GROWTH AND MAINTENANCE OF DOGS FED AMINO ACIDS AS THE SOURCE OF DIETARY NITROGEN

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ONE FIGURE

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Several investigators have studied the replacement of protein in diets of dogs with protein hydrolysates or amino acid mixtures. In 1943 Madden, Carter, Kuttus, Miller and Whipple reported that the 10 "essential" amino acids of Rose ('38) and Rose and Rice ('39) satisfied the requirements of the dog for plasma protein production, weight maintenance, and nitrogen balance. Various partial hydrolysates of protein and a mixture of L- and DL-amino acids have been shown to maintain nitrogen balance when given to dogs by intravenous infusion (Cox and Mueller, '46; Kade, Houston, Krauel and Sahyun, '46; Frost and Risser, '46). Recently, however, it was reported by Miller, Robscheit-Robbins and Whipple ('47) that their doubly depleted dogs "practically always" lost weight when amino acids were supplied in place of protein. Although these authors suggest that an unidentified deficiency may have been responsible for this effect, their food consumption data indicate that the weight loss was associated with a loss of appetite which resulted in an inadequate caloric intake.

The experiments reported in the present paper were designed to determine whether peptide-free amino acid mixtures

satisfy the requirements of the dog for growth and maintenance, and whether exclusion of peptides from the diet results in pathological changes. The amino acid mixtures which were supplied as the sole source of nitrogen were derived from casein and were of the VujN type employed in previous studies (Silber, Seeler and Howe, '46; Smyth, Levy and Sasichak, '47; Eckhardt, Cooper, Faloon and Davidson, '48).

EXPERIMENTAL

Experiment I. Adult dogs

For a period of two weeks, 6 adult mongrel dogs were fed a protein-free basal diet (Silber, Seeler and Howe, '46) plus 1.5 gm of casein per kilogram per day to supply a total of 80 cal. and 210 mg nitrogen per kilogram per day. At the end of this "conditioning" period, two of the dogs were fed amino acid mixture No. 1 (table 1) in place of the casein, two continued to receive casein, and two were supplied neither protein nor amino acids. The daily intake of nitrogen and calories was kept constant throughout the experiment. The amino acid mixture, which was prepared in a 10% aqueous solution, was added to the diet after lyophilization. After the mixture had been supplied in the diet for 40 days, it was given to the same two dogs intravenously (6 mg N per kilogram per minute) for an additional 24 days. Urines and feces were collected continously, and pooled 8-day samples were assayed for total nitrogen and α-amino nitrogen. Blood samples were taken every 8th day for serum protein determinations and for hematological study. Larger samples of blood were taken at the beginning and at the end of the experiment for determination of certain blood constituents (for the methods used see Silber, Porter, Winbury and Clark, '47; for plasma CO_2 capacity see Van Slyke and Cullen, '17). Total fat, nitrogen, and glycogen (Sahyun, '31) contents of the livers were determined on samples taken at autopsy. A number of tissues were fixed in 10% neutral formalin for histological study.

The composition of the amino acid mixture (No. 1) and the average daily intake of each essential amino acid are recorded in table 1. Since preparation of the amino acid mixture involved complete acid hydrolysis of casein, followed by isolation and purification of groups of amino acids, and finally, recom-

TABLE 1

L-Amino acid composition[1] of mixtures and average daily intake of essential amino acids

	ADULT DOGS		PUPS	
	Mixture no. 1	Intake	Mixture no. 2[2]	Intake
	mg/ml	*mg/kg/day*	*mg/ml*	*mg/kg/day*
Arginine	7.5	78	2.6	173
Histidine	3.2	46	4.1	272
Isoleucine	6.9	98	7.9	525
Leucine	14.2	202	16.4	1,090
Lysine	9.8	139	11.4	760
Methionine	6.0[3]	121	3.8	252
Phenylalanine	5.9	84	4.0	266
Threonine	2.3	33	1.9	126
Tryptophan	0.75[4]	11	0.9[4]	60
Valine	6.3	90	6.8	450
Glycine	22.6		23.6	
Total N	14.9		14.7	
Amino N	12.7		12.1	
Total solids	107.3		103.0	

[1] These preparations contain about 10 mg/ml of non essential amino acids such as serine, proline, alanine, glutamic acid, and aspartic acid.

[2] Contains about 10% lactic acid.

[3] Contained 2.5 mg of D methionine in addition.

[4] Added in the form of DL tryptophan.

bination of the basic fraction and the monoaminomonocarboxylic fraction, the absence of peptides was assured. The mixture gave a negative biuret test and showed no increase in α-amino nitrogen after further acid hydrolysis.

Results

The 4 dogs which received casein or amino acid mixture No. 1 maintained nitrogen balance and body weight. Serum pro-

TABLE 2

Body weights, serum protein concentrations and nitrogen balance in adult dogs

DOG NO.	N SOURCE	INITIAL			40 DAYS			64 DAYS		
		Wt.	Protein	Albumin	Wt.	Protein	Albumin	Wt.	Protein	Albumin
		kg	*gm %*	*gm %*	*kg*	*gm %*	*gm %*	*kg*	*gm %*	*gm %*
721	Casein	7.6	5.2	3.0	8.0	5.7	3.0	8.1	5.4	2.8
798	Casein	7.6	5.2	3.0	7.7	5.5	3.1	7.7	5.6	3.4
753	Amino acids	10.1	5.8	2.5	10.6	5.7	3.2	10.0	5.2	2.9
818	Amino acids	7.2	4.8	2.7	7.6	5.2	3.4	7.4	5.0	2.9
828	None	7.5	5.0	2.8	6.8	4.1	2.0	6.3	4.2	1.9
853	None	13.2	5.3	3.3	10.8	4.2	2.3	9.5	3.8	2.0
		Nitrogen intake and excretion, mg per kg initial body weight per day								
		CONTROL PERIOD[1]								
		Intake	Urine	Feces	*Intake*	*Urine*	*Feces*	Intake	Urine	Feces
721	Casein	210	219	21	210	192	29	210	172	18
798	Casein	209	208	25	209	156	37	209	124	23
753	Amino acids	216	181	22	215	187	16	215	182	14
818	Amino acids	210	198	27	216	163	23	216	170	18
828	None	208	183	26	5	74	15	5	42	10[2]
853	None	210	205	20	5	83	20	5	44	10[2]

[1] Eight-day period.

[2] Lower values here due to decreased food consumption of depleted dogs.

tein concentrations remained essentially unchanged (table 2). Body weight, as well as total serum protein and albumin concentrations, showed a progressive decrease in the protein-depleted dogs.

The fraction of administered nitrogen excreted as the sum of urea, ammonia and amino acid nitrogen was approximately the same whether the amino acids were given orally or intravenously. Hence, nitrogen balance values were independent of the method of administration, although 15 to 20% of the infused amino acids appeared in the urine. When the nitrogen was supplied orally, 75 to 80% was excreted in the form of urea and ammonia; after intravenous infusion, only 63% was excreted in this form. No significant fraction of the amino acid nitrogen administered by either route appeared in the feces, but about 5% of the casein was accounted for as fecal nitrogen (table 2).

During the period of oral feeding, the plasma protein concentrations of both casein- and amino acid-fed dogs increased slightly; after infusion of the amino acids a slight decrease was noted. The plasma albumin concentrations of the dogs fed amino acids in the diet increased, whereas the globulin concentrations decreased (table 2).

The dogs which received amino acids had 11% more hepatic protein, whereas the depleted dogs had 21% less than the casein-fed animals, as indicated by total nitrogen analyses.[1] Furthermore, the ratio of liver weight to body weight was 30% greater in amino acid-fed dogs than in the casein-fed animals.

There were no significant differences between the amino acid- and casein-fed animals in the ratio of plasma cholesterol ester to total cholesterol (0.77), in plasma fibrinogen (265–295 mg%), in serum alkaline phosphatase (1.2–1.8 Bodansky units), in plasma CO_2 capacity (50–54 volumes %), in liver total lipid (9.4–11.5%), in liver glycogen (6.8–7.5%), in plasma

[1] Casein-fed dogs 73 and 74 mg N/100 gm body weight; amino acid-fed dogs 80 and 83 mg N/100 gm; and protein-depleted dogs 52 and 64 mg N/100 gm, respectively.

NPN (22–29 mg%), in plasma glucose (115 mg%), or in plasma creatinine (1.2–1.3 mg%).

Table 3 shows the pertinent hematological data obtained from these dogs. By the 64th day of test, both dogs on the protein-free diet had developed a normocytic anemia, evidence of which had appeared in one of the dogs as early as the 40th

TABLE 3

Hematological data on adult dogs

DIET	DOG NO.	DAY	ERYTHROCYTES	HEMOGLOBIN	HEMATOCRIT	LEUKOCYTES PER MM³
			millions/mm³	*gm/100 ml*	*vol. %*	*no.*
Casein	721	0	6.3	14.8	49	7,880
		40	6.4	14.0	50	6,800
		64	5.7	14.3	50	8,280
Casein	798	0	6.7	15.0	50	5,040
		40	6.3	15.0	50	7,040
		64	6.2	16.5	53	9,040
Amino acids	753	0	6.4	15.4	52	9,640
		40	6.1	14.0	51	8,040
		64	6.4	14.5	55	12,400
Amino acids	818	0	6.3	15.5	50	8,760
		40	6.9	15.4	52	8,080
		64	6.2	15.0	50	6,920
Protein-free	828	0	6.4	14.5	48	6,320
		40	6.2	13.0	43	6,320
		64	4.8	11.3	38	6,200
Protein-free	853	0	6.8	14.3	49	5,320
		40	6.5	14.5	47	6,920
		64	5.1	11.3	37	7,720

day. The dogs which were given the amino acid mixture, initially in the diet and later by vein, maintained normal erythrocyte values, as did the casein-fed animals. No significant changes were observed in the total or differential leukocyte counts of any of the dogs.

Gross and microscopic examination of tissues revealed no definite evidence of toxic effects due to the administration of

the amino acid mixture. The liver of one dog on the protein-free diet showed a moderate amount of lipid in the periportal regions. Only a small quantity of hepatic lipid was seen in the other dog on this diet. No lipid was observed in the livers of dogs given casein or amino acids in the diet. The presence of sudanophilic material in Henle's loops and of occasional interstitial round cell collections was noted in the kidneys of dogs of all three groups, and can be ascribed to age and previous history rather than to the specific treatment received during the experiment. An unexplained finding in the kidney of one amino acid-dosed dog consisted of lipid globules present in the glomeruli at the vascular pole. On the average, the number and activity of Malpighian follicles in the spleen, as well as the spleen weight-body ratio, were less in the protein-depleted dogs than in the animals of the other two groups. The amount of tissue hemosiderin varied, but one animal on the protein-free diet showed a rather marked hemosiderosis of liver, spleen and bone marrow. No significant histopathological changes were noted in the heart, lungs, adrenal glands, thyroid glands, pituitary gland, bone marrow, pancreas, urinary bladder, skin, sex organs or gastrointestinal tract of any dog.

Experiment II. Puppies

A litter of 12-week-old Beagle pups was divided into three groups of two each. One group was fed a diet containing 20% of amino acid mixture No. 2 (table 1) ad libitum, the second received an identical amount of diet but with casein (14.7% N) in place of the amino acids, and the third received the protein-free diet only. The 10% solution of amino acids was concentrated under reduced pressure to about 15% of its original volume before being mixed with the diet. After one month, the pups fed the protein-free diet had lost weight and their food consumption was almost negligible. At this time they were supplied the amino acid diet ad libitum.

Every week the pups were weighed and then bled for hematological study. Urines were collected after one month and

again after three months for urinary analyses. At the termination of the experiments, bromsulfalein retention was determined and blood samples were taken for analyses. After receving casein or amino acids for a total of 12 weeks, the dogs were sacrificed and tissues were taken for pathological examination.

Results

The pups given casein or amino acids exhibited comparable rates of growth (fig. 1). The body weights of the protein-depleted pups decreased during the first 4 weeks but showed

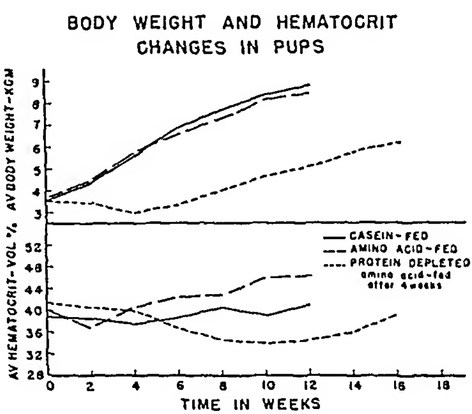

Figure 1

gradual and consistent gains when the amino acid diet was substituted for the protein-free diet. In 12 weeks the body weights of these dogs increased 100%.

No significant differences between the amino acid-fed and casein-fed pups were found in the ratio of plasma cholesterol ester to total cholesterol (0.76–0.77), in plasma fibrinogen (285–290 mg %), in alkaline serum phosphatase (3.6–3.8 Bodansky units), in plasma NPN (37–38 mg %), in plasma glucose (105 mg %), in plasma creatinine (1.4–1.6 mg %), in serum albumin (3.6–3.8 gm%) or in bromsulfalein retention (10–

15% at 5 minutes, less than 5% after 15 minutes). No significant changes were revealed by the urinary analyses.

At the end of the experiment, the plasma protein concentrations of the casein-fed pups were 5.5 and 5.7 gm %, whereas the amino acid-fed animals had concentrations of 4.8 and 5.1 gm %. However, plasma albumin was increased by amino acid feeding, resulting in albumin/globulin ratios of 2.3 and 2.8 in these dogs in contrast to 1.7 and 2.4 in the casein-fed animals. Analyses of the livers of two of the dogs showed that the total hepatic N was 16.5% greater on a body weight basis after amino acid feeding.

During the 12-week experimental period the casein-fed pups showed an over-all increase in erythrocyte count, hemoglobin concentration and hematocrit values. The pups getting the amino acids, after a slight initial decrease in these blood values, showed an increase which, by the end of the test, was greater than that of the casein-fed controls. A progressive anemia developed in the pups fed the protein-free diet. Even after amino acids were added to their diet, these animals continued to show anemia. However, the beneficial effects of amino acid therapy became evident after the 10th week, and by the 16th week recovery from the anemia was well advanced. The total and differential leukocyte counts in all three groups of animals fluctuated within the normal limits for this species.

Gross and microscopic examinations of tissues from the pups showed, as the only noteworthy change, the presence of moderate amounts of lipid in the periportal regions of the livers of animals which had received the protein-free diet. The ratio of spleen weight to body weight was lowest in this group. The glycogen content of the livers, as shown by Best's carmine stain, was comparable in all animals. No histopathologic changes related to dietary treatment were found in the kidneys, heart, lungs, spleen, thymus, adrenal glands, thyroid glands, pituitary gland, pancreas, urinary bladder, skin, bone marrow, sex organs or gastrointestinal tract of any of the dogs.

DISCUSSION

The growth-promoting properties of amino acids for rats have been the subject of numerous investigations, notably those of Rose ('38). Although similar growth studies in dogs have not been reported, nitrogen balance data are cited by Rose and Rice ('39) to show that the amino acid requirements of the adult dog are qualitatively similar to those of the rat. The experiments of Madden, Carter, Kuttus, Miller and Whipple ('43) support this contention. The feeding of peptide-free amino acid mixtures containing the 10 "essential" amino acids and about 35% of non-essential amino acids has been shown, in the present experiments, to maintain adult dogs and to promote the growth of pups, when supplied with a basal diet also free of peptides.

It is of interest to note that amino acid feeding favored hepatic protein and plasma albumin production in both young and adult dogs. The plasma A/G ratios of the amino acid-fed animals were 25% greater, on the average, than those of the casein controls. Similarly, the hepatic protein was 11 to 16% greater. This difference between the utilization of casein and that of the amino acid mixtures is presumably due to differences in amino acid composition or availability.

Both pups and adult dogs fed a protein-free diet developed anemia. When amino acids were added to the diet of the pups, a delayed but definite increase occurred in erythrocyte, hemoglobin, and hematocrit values. Correction of the anemia was no doubt masked initially by the more rapid correction of the dehydration suffered by these animals as a result of inanition.

In studies of the nutritional value of amino acid mixtures, the adequacy of the basal diet and an optimum caloric intake must be guaranteed. The adequacy of our basal diet for the growth and maintenance of dogs, when casein is included as the dietary protein, has been demonstrated previously in experiments of 2½ to 4½ years' duration (Silber, '44; Seeler and Silber, '45). The daily caloric intake of the adult dogs was

kept constant throughout the experiments, on the basis of 80 cal. per kilogram of initial, fasted, body weight. The caloric intake of the pups ranged from between 225 and 110 cal. per kilogram per day.

When the pups were first offered the amino acid diet, they ate it rapidly and vomitted shortly thereafter, but within several days they learned to consume the diet in small portions and succeeded in retaining sufficient amounts for growth. It is important to note that if the pups had not learned to eat the diet in small portions, it would have been considered inadequate in the usual nutritional sense.

Since both dogs and rats show evidence of a physiological disturbance (nausea or diarrhea) when amino acids or protein hydrolysates are given orally, it seems reasonable to assume that this may be related to limitation of the voluntary intake of amino acid-containing diets and may therefore explain, at least in part, the inability of these animals to grow as rapidly on amino acid diets as on high quality protein diets supplied ad libitum. In general, if an animal fails to consume sufficient diet to satisfy his caloric needs, the diet may appear to be unsatisfactory for growth and nitrogen balance in spite of the presence of all essential factors.

There is no evidence that the physiological disturbance following rapid ingestion of amino acid mixtures is due to unknown toxic factors. Diarrhea has been observed in rats given a variety of protein digests, both enzymatic and acid. It seems likely that the physiological disturbance is due to a nonspecific osmotic effect of the amino acids, and would therefore depend upon the degree rather than the method of hydrolysis.

The data reported in this paper do not exclude the possibility that proteins may contain factors other than amino acids which are beneficial to the animal. Nevertheless, there is no doubt that the growth and maintenance requirements of the dog can be satisfied by feeding a diet containing amino acids as the sole source of nitrogen.

SUMMARY

1. Two adult dogs were fed a peptide-free mixture of amino acids as the sole source of nitrogen, orally for 40 days and intravenously for an additional 24 days. Body weights, serum protein concentrations, and nitrogen balace were maintained. No biochemical, hematological or pathological evidence of toxicity or deficiency was observed.

2. Two pups were fed for 12 weeks a diet containing a peptide-free mixture of amino acids as the sole source of nitrogen. Their average gain in weight was 0.40 kg per week. Pair-fed animals receiving a casein diet gained 0.43 kg per week. There was no indication that the amino acid mixture produced toxic effects, other than an initial transient nausea.

3. Peptides do not appear to be essential for the growth or maintenance of dogs.

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THE INFLUENCE OF COPROPHAGY ON THE BIOTIN AND FOLIC ACID REQUIREMENTS OF THE RAT [1]

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The importance of the intestinal microflora in the nutrition of animals has been demonstrated by many groups of workers. While the animal may benefit to a considerable extent from the activity of the organisms present in the tract, this phenomenon introduces an unknown and complicating factor in the study of the nutritional requirements of the animal. A portion of the metabolic products of the intestinal flora may be absorbed directly from the intestinal tract, and another portion may be obtained by the animal through the ingestion of its fecal matter, a habit known as coprophagy. This phenomenon was first observed by Osborne and Mendel ('11), and it has been studied by many groups of investigators (Steenbock et al., '23; Smith et al., '25; Salmon, '25). Although it was shown by Guerrant, Dutcher and Tomey ('35) that the effects

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of coprophagy were more marked when the carbohydrate of the diet was dextrin, they also recognized some effects due to coprophagy when the diets contained sucrose.

Geyer et al. ('47) recently described the construction of a very satisfactory type of coprophagy-preventing cage. Essentially this cage consists of a horizontal expandable mesh wire tube with provisions for food cup and water bottle on the front end. It allows the rat limited movement without any access to its excreta. In the present paper data are presented on the growth rate and vitamin content of the livers of rats raised in the tube type of cage or in ordinary screen-bottom cages. Some of the diets fed were supplemented with sulfonamide.

EXPERIMENTAL AND RESULTS

Twenty-one-day-old male rats of the Sprague-Dawley strain weighing 40–45 gm were used in all experiments. The rats were housed in individual metal cages (square or tubular as specified). Feed and water were provided ad libitum. The composition of the basal diets is given in table 1. The composition of the water soluble vitamin supplements is listed under the respective headings in table 2. The diets were mixed approximately once weekly and were stored in a refrigerator. In the first experiment comparable groups were housed in square screen-bottom cages and in coprophagy-preventing tube cages, and the effect of supplementing the diets with two different mixtures of water soluble vitamins was studied. Mixture "A" contained thiamine, riboflavin, pyridoxine, calcium pantothenate and choline. Mixture "B" differed from "A" in that it contained in addition the following 5 vitamins: Niacin, folic acid, biotin, *p*-aminobenzoic acid and inositol (table 2). This comparison was carried out with basal diets I and II containing corn oil and butterfat respectively as the sole source of dietary fat (table 1). Thus, there were 8 groups, each containing 12 rats. The results of this experiment are presented in table 3.

It will be noted that confinement in the tube cages reduces the growth of all the rats when compared to that of their mates on the same diets in the square cages. However, attention is directed to the differences between the groups within each type of cage. While the 5 additional vitamins present in supplement "B" improved the growth of the rats on the butterfat and the corn oil diets by 13 and 11% respectively when they were housed in the square cages, the growth increments for the rats in the tube cages were 30 and 29% respectively. There

TABLE 1

Composition of the basal rations[1]

CONSTITUENTS	BASAL RATION NUMBER I	II	III	IV
Casein[2]	20	20	20	20
Salts IV[3]	4	4	4	4
Corn oil[4]	28		28	
Butterfat[5]		28		28
Sucrose	48	48		
Lactose			48	48

[1] To each ration were added the following fat soluble vitamins per 100 gm ration: 210 µg 2-Me-1,4-naphthoquinone; 14 µg crystalline irradiated ergosterol; 560 µg β-carotene in the form of a preparation containing 90% β-carotene and 10% α-carotene; and 2.24 mg α-tocopherol.

[2] Extracted for three two-hour periods with boiling alcohol.

[3] Hegsted et al. ('41).

[4] Mazola brand, Corn Products Refining Company.

[5] The butterfat was prepared by melting fresh sweet butter obtained from the University dairy, followed by decantation and filtration.

was no apparent difference in growth due to the type of fat used under the conditions of this experiment.

The second experiment was planned to ascertain which of the 5 additional vitamins was involved in the growth improvement observed in the first experiment. Seven groups of 12 rats each were placed in tube cages and fed basal diet I containing corn oil. Each group received one of the following water soluble vitamin supplements (see table 2): "A," "B" minus folic acid, "B" minus biotin, "B" minus nicotinc acid, "B" minus *p*-aminobenzoic acid, "B" minus inositol and

TABLE 2

Composition of water soluble vitamin mixtures (all values in mg/100 gm ration)

	"A"	"A" PLUS FOLIC ACID	"A" PLUS BIOTIN	"A" PLUS FOLIC ACID PLUS BIOTIN	"B"	"B" MINUS FOLIC ACID	"B" MINUS BIOTIN	"B" MINUS NICOTINIC ACID	"B" MINUS p-AMINOBENZOIC ACID	"B" MINUS INOSITOL	LOW
Thiamine	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.10
Riboflavin	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.70	0.15
Pyridoxine	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.60	0.15
Calcium pantothenate	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	0.75
Choline hydrochloride	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00	150.00
Folic acid		0.20		0.20	0.20		0.20	0.20	0.20	0.20	.
Biotin			0.02	0.02	0.02	0.02		0.02	0.02	0.02	.
Nicotinic acid	.			.	0.60	0.60	0.60		0.60	0.60	..
p-Aminobenzoic acid		.	.		1.50	1.50	1.50	1.50	.	1.50	...
Inositol	..	..	.	.	5.00	5.00	5.00	5.00	5.00	...	...

"B." Seven comparable groups were placed in the square cages. Again all the groups in the square cages grew better than their mates on the same diets in the tube cages. In order to bring out the relative improvement in growth on each supplement in each type of cage, all groups in one type of cage are compared to the negative control, group "A," in the same type of cage, and the values in table 4 represent the increment in per cent growth gain (6-week period) over that of the "A" group. The absolute growth values for the "A" group in the two types of cages are also given.

TABLE 3

Results of experiment 1. (Average[1] gain in gm/6 weeks, 12 rats per group)

Basal ration Dietary fat Water soluble vitamin supplement		I CORN OIL "A"	I CORN OIL "B"	II BUTTERFAT "A"	II BUTTERFAT "B"
Tube cage	gm gain/6 weeks	125 ± 3.87	161 ± 4.73	121 ± 3.38	157 ± 3.83
	growth increment over supplement "A"		+ 29%		+ 30%
Square cage	gm gain/6 weeks	185 ± 8.8	207 ± 6.9	181 ± 5.05	206 ± 4.93
	growth increment over supplement "A"		+ 11%		+ 13%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

In this experiment the growth stimulation due to the "B" supplement containing all the 5 additional vitamins is again demonstrated to a considerably greater degree in the tube cage (25%) than in the square cage (9%).

In the tube cages, the weight gain of rats on the diets containing supplements "B" minus folic acid and "B" minus biotin were small (9 and 3% respectively) compared to those on the diet containing the "B" supplement. On "B" minus *p*-aminobenzoic acid the gains were intermediate in the tube cages (15% over those of group "A") while in the square

cage they were greater even than on the full "B" supplement. Furthermore, a number of the rats in the tube cages not supplemented with biotin showed the "spectacle eye" symptom.

After finding that growth was suppressed by removing either folic acid or biotin from the "B" supplement, it was of interest to study the effect of these vitamins on growth when they were added to the "A" supplement. In the third experiment 5 comparable groups of 6 rats each were placed in tube cages and fed basal diet I containing corn oil. Each group received one of the following water soluble vitamin

TABLE 1

Results of experiment 2. (Average[1] gains in comparison to gains on supplement "A" in each type of cage, 12 rats per group)

VITAMIN SUPPLEMENT	TUBE	SQUARE
"A" (gm gains)	120.5 ± 5.6	172.0 ± 6.8
	Growth increment as compared to "A"	Growth increment as compared to "A"
"B" minus folic acid	+ 9%	+ 6%
"B" minus biotin	+ 3%	+ 6%
"B" minus niacin	+ 26%	+ 9%
"B" minus *p*-aminobenzoic acid	+ 15%	+ 13%
"B" minus inositol	+ 22%	+ 9%
"B"	+ 25%	+ 9%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

supplements: "A," "A" + folic acid, "A" + biotin, "A" + folic acid + biotin, and "B" (table 2). Similar groups of 6 rats each were placed in the square cages. The results of experiment 3 are given in table 5. The growth of rats in the tube cages was improved 13% by addition of folic acid (supplement "A" + folic acid) and 8% by the addition of biotin (supplement "A" + biotin). When these were added together (supplement "A" + folic acid + biotin) the improvement was 23%, which was comparable to the growth obtained with the addition of the full supplement "B" (22%). In the square cage

the increments were neither as large nor as regular as those observed in the tube cage.

In the 4th experiment studies were carried out to observe the effect on rats in tube cages of corn oil and butterfat diets containing either sucrose or lactose as the source of carbohydrate (diets I, II, III, and IV). The effects of folic acid and biotin were tested with each combination by adding vitamin supplements "A," "A" + folic acid, "A" + biotin, and "A" + folic acid + biotin. Also, supplement "B" containing the 10 B-complex vitamins was added to lactose diets III and IV.

TABLE 5

Results of experiment 3. (Average[1] gains in comparison to gains on supplement "A" in each type of cage, 6 rats per group)

VITAMIN SUPPLEMENT	TUBE	SQUARE
"A" (gm gain)	122 ± 7.3	182 ± 7.8
	Growth increment as compared to "A"	Growth increment as compared to "A"
"A" plus folic acid	+ 13%	+ 8%
"A" plus biotin	+ 8%	+ 4%
"A" plus folic acid and biotin	+ 23%	+ 2%
"B"	+ 22%	+ 3%

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\frac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

Thus a total of 18 groups of 6 rats each were placed in tube cages and fed the above diets. The average gains during the 6-week period are given in table 6.

The results of this experiment confirm those obtained in the previous studies on the effects of folic acid and biotin. On the sucrose diet the differences between the comparable groups were too narrow to allow any statement as to the superiority of one fat over another. On the lactose diets, however, wider differences were observed among all the comparable groups on corn oil and butterfat.

Neilsen and Elvehjem ('42) showed that sulfasuxidine increases the dietary requirement of the rat for biotin and folic acid. In experiment 5 the effect on the vitamin content of the liver of preventing coprophagy was compared with that due to depression of intestinal synthesis by the addition to the diet of 0.5% sulfasuxidine at the expense of sucrose. For comparison, rats were also raised in square cages without sulfonamide. Under each of these conditions butterfat was compared with corn oil. In this experiment low levels of thiamine, riboflavin, pyridoxine, and calcium pantothenate were used (last column of table 2). The experiment was started with 6 groups of 6 rats each, but during its course one rat died in each of the tube cage groups and therefore all the values are based on 5 rats per group. Three rats in the tube cages and 4 in the sulfa-fed groups showed the "spectacle eye" condition. Three rats in the latter groups showed bilateral hair loss on sides and hind quarters. This condition was reported also by Nielsen and Black ('44), who found it could be prevented by the addition to the diet of an inositol supplement.

TABLE 6

Results of experiment 4. (Average[1] gain in gm/6 weeks, 6 rats per group)

BASAL RATION	I	II	III	IV
DIETARY CARBOHYDRATES DIETARY FAT	SUCROSE CORN OIL	SUCROSE BUTTERFAT	LACTOSE CORN OIL	LACTOSE BUTTERFAT
Supplement				
"A"	116 ± 7.49	119 ± 5.20	59 ± 5.29	76 ± 5.29
"A" + folic acid	129 ± 5.61	138 ± 5.26	75 ± 3.16	82 ± 4.76
"A" + biotin	142 ± 3.94	131 ± 3.77	66 ± 5.09	85 ± 3.81
"A" + folic acid + biotin	144 ± 4.12	136 ± 4.94	81 ± 8.71	90 ± 5.46
"B"			83 ± 7.46	95 ± 5.68

[1] Including the standard error of the mean as calculated from $s_x = \sqrt{\frac{\Sigma(X - x)^2}{n(n-1)}}$.

TABLE 7

Results of experiment 5. (Average[1] gains in gm/6 weeks, and levels of folic acid, biotin and niacin in the livers of the rats, 5 rats per group)

BASAL RATION DIETARY FAT	SQUARE CAGE I corn oil	SQUARE CAGE II butterfat	TUBE CAGE I corn oil	TUBE CAGE II butterfat	SQUARE CAGE AND 0.5% SULFASUXIDINE I corn oil	SQUARE CAGE AND 0.5% SULFASUXIDINE II butterfat
Growth in gm/6 weeks	92 ± 3.5	114 ± 9.81	53 ± 5.8	60 ± 7.8	68 ± 8.2	59 ± 7.6
μg Folic acid/gm liver	0.43 ± 0.075	0.34 ± 0.068	0.20 ± 0.033	0.21 ± 0.024	0.09 ± 0.016	0.08 ± 0.026
μg Biotin/gm liver	0.82 ± 0.078	0.72 ± 0.081	0.36 ± 0.049	0.38 ± 0.027	0.23 ± 0.026	0.29 ± 0.028
μg Niacin/gm liver	119 ± 7.73	121.5 ± 5.74	120.8 ± 6.03	118.2 ± 5.17	139 ± 5.4	134.6 ± 3.69

[1] Including the standard error of the mean as calculated from $s_{\bar{x}} = \sqrt{\dfrac{\Sigma(X - \bar{x})^2}{n(n-1)}}$.

At the end of the 6-week growth period the rats were killed by decapitation and the livers removed for vitamin analysis. The method recommended by Olson et al., ('48) was followed for the liberation of the folic acid. Samples of the liver homogenates were incubated at pH 7.0 for 4 to 5 hours, after which the enzymatic reaction was stopped by immersing the tubes in boiling water for three minutes. The folic acid content was determined by using *Streptococcus faecalis* (American Type Culture Collection no. 8043) and the turbidity method of Luckey, Briggs and Elvehjem ('44), in which the medium was modified by the addition of Salts B (Teply and Elvehjem, '45).

The biotin content of the livers was determined by a modification of the method of Wright and Skeggs ('44) in which turbidity was used as the criterion in measuring the growth of *Lactobacillus arabinosus*. For the nicotinic acid assay *L. arabinosus* and the method recommended by Snell ('47) were used.

Growth gains during the 6-week period and the levels of the three vitamins in the livers are given in table 7. Considerably higher levels of biotin and folic acid were found in the livers of rats from the square cages than in those from the tube cages or those fed sulfasuxidine. Of the last two groups, lower levels of these two vitamins were found in the livers of the sulfa-fed animals than in those from the tube cages. This was true on both the corn oil as well as on the butterfat diets. In contrast, the niacin levels of the livers of all groups seem to be similar.

DISCUSSION

It is evident from tables 3, 4, 5 and 6 that under our experimental conditions both folic acid and biotin produce a growth response in rats on diets containing 28% fat and 48% sucrose or lactose. Heretofore the requirement for these two factors by the rat has been clearly demonstrated only when some partially toxic material like a sulfonamide or avidin was incorporated in the deficient diet. While the better growth due to the addition of these two vitamins to the diet is to a certain degree demonstrated in the square cage, their presence in the

diet seems of relatively greater value for the rats deprived of coprophagy. Furthermore, the "spectacle eye" symptom of biotin deficiency (Nielsen and Elvehjem, '41) was observed only in the rats kept in the tube cages.

It would seem that the practice of using a raised screen floor cage, like our square cage, would entirely eliminate coprophagy. However, Geyer et al. ('47) often observed that some rats kept under such conditions obtained feces pellets as they were excreted. Since this habit is more common in some rats than in others, it leads to greater variability in growth data obtained with rats maintained in square cages when the limiting factors are such as can be obtained through coprophagy. Some of the early observers of coprophagy (Steenbock, Sell and Nelson, '23; Guerrant and Dutcher, '32) also noted the variability in the experimental results due to this phenomenon. This is best demonstrated in table 4, where the results of the square cage groups are most irregular.

The growth increment (9%) due to vitamin supplement "B" minus folic acid which contains biotin (table 4) checks well with that (8%) due to vitamin supplement "A" + biotin (table 5). The presence of the three additional vitamins, *p*-aminobenzoic acid, inositol and niacin, in experiment 2 (table 4) seems to exert no apparent effect on growth. However, an inspection of the growth increment (3%) due to "B" minus biotin which contains folic acid (table 4) shows it to be lower than that (13%) due to supplement "A" + folic acid (table 5), which also contains folic acid but no *p*-aminobenzoic acid, inositol or nicotinic acid. This might be due to the variations regularly encountered in such biological experiments. On the other hand, a possible explanation is that the additional vitamins (*p*-aminobenzoic acid, inositol and nicotinic acid) may accentuate the growth of microorganisms which tend to be folic acid users and thus deprive the animal of the folic acid obtained in the tube cage by absorption from the intestinal tract.

The liver biotin and folic acid values represented in table 7 should not be considered a quantitative estimation of the intestinal synthesis of these two vitamins, their absorption di-

rectly from the ceca, or the amount obtained by the rat through his habit of coprophagy, because (a) there is some synthesis in the gastrointestinal tract in spite of the sulfa drug, (b) part of the vitamins synthesized by some microorganisms are destroyed by others whether sulfa is present or not, and (c) the quantitative aspects of these phenomena differ too greatly among individual rats to allow us to make conclusive statement on the basis of the small number of rats used in this experiment. However, the values found are very interesting and significant in demonstrating these phenomena at least qualitatively under the three conditions: Square cage, tube cage and square cage with sulfasuxidine.

Salmon ('47) found that high levels of fat tended to counteract a deficiency of niacin. He believes that this is a nicotinic acid-sparing effect of fat. Hankes et al. ('48) reported similar observations. In addition to the possibility of the sparing effect, they suggest also the possibility of better synthesis or decreased destruction of niacin by the intestinal flora when a high fat ration is fed.

Krehl et al. ('45) showed that tryptophan can replace nicotinic acid in counteracting the growth retardation caused by corn grits in a low protein ration. Rosen et al. ('46) concluded from their excretion studies that tryptophan may be the important precursor of nicotinic acid synthesis in the rat. Similar observations were made also by Singal and his group ('46). Our data on the liver content of nicotinic acid seem to be in line with these studies. The niacin values shown in table 7 indicate that neither the type of dietary fat (28% level) nor the abiltiy or inability of the rat to consume its feces affected the amount of liver niacin, under our conditions. Rather, it seems to have been determined by the 20% casein the rats received in their diets, which also contained 28% of fat.

SUMMARY

1. Rats fed purified diets and kept in coprophagy-preventing tube cages grew significantly better when folic acid and biotin were added to the diet than when these vitamins

were omitted. Inositol, *p*-aminobenzoic acid, and niacin did not affect the growth rate of these rats. Biotin and folic acid produced only slight and irregular improvement in the growth of rats kept in ordinary screen-bottom cages.

2. The separate effects of coprophagy-prevention and the addition of sulfasuxidine were reflected also in the biotin and folic acid content of the liver, but not in the nicotinic acid content.

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ORAL MANIFESTATIONS IN RATS FED SYNTHETIC DIETS DEFICIENT IN PANTOTHENIC ACID AND BIOTIN[1]

I. METHODS AND GENERAL GROSS SYMPTOMS

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TWO FIGURES

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The purpose of this study was to observe the oral lesions produced by a synthetic diet in which calcium pantothenate, panthenol, biotin and folic acid were the experimental variables. The replacement effects of these agents were noted by their use singly and in various combinations. Skin manifestations and weights were also considered.

Ziskin, Stein, Gross and Runne ('44, '45, '47) reported the production of mouth ulcers, hyperkeratinization of the oral mucous membrane, decolorization of the incisors, and marked gingival and periodontal necrosis in rats by the addition of zinc carbonate to a diet which was suboptimum in pantothenic acid. Similar results were obtained by them in rats fed a synthetic diet totally deficient in pantothenic acid but ade-

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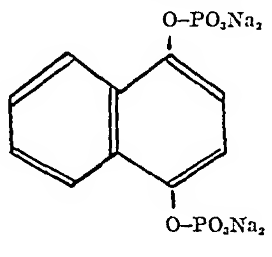
quate in every other respect, although not as many animals were affected. Wainwright and Nelson ('45) reported generally comparable results in rats on a pantothenic acid deficient diet without the addition of zinc carbonate.

TABLE 1

Composition of basal diets

CONSTITUENTS	BASAL DIET 1	BASAL DIET 2	VITAMIN SUPPLEMENTS ADDED TO COMPLETE BASAL DIETS	AMOUNT PER ANIMAL PER DAY
	%	%		
Casein, vitamin-free	24		Thiamine hydrochloride	20 μg
Sucrose	64		Pyridoxine hydrochloride	20 μg
Egg white		30	Riboflavin	40 μg
Dextrin		55	Nicotinic acid (niacin)	1 mg
Crisco	5	8	Choline chloride	4 mg
Cod liver oil	3	3	Para-aminobenzoic acid	250 μg
Salt mixture	4	4	*i*-Inositol	500 μg
			Vitamin E [1]	400 μg
			Vitamin K [2]	80 μg

[1] d-l-Tocopherol, phosphoric acid disodium; synthetic water soluble vitamin E.
[2] Synkayvite — synthetic vitamin K (water soluble).



EXPERIMENTAL

Twenty groups of rats, 18 of 10 animals each and two of 16 animals each, were used in this study. There were equal numbers of males and females in each group. The weanling rats were maintained in litters with the mother until they attained the desired weight, which was 40 ± 2 gm, at which time they were weaned and placed on the synthetic diets.

The rats were housed in individual cages with wire-mesh bottoms. The wire-mesh reduced the practice of coprophagy,

but did not entirely prevent it. Water and the respective basal diets as shown in table 1 were supplied in unlimited quantities.

The preparation of the vitamin supplements (table 1) which completed the basal diets, and the manner of feeding these

TABLE 2

Combinations of experimental variables added to basal diets

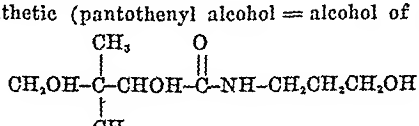
DIETS	GROUP [1]	BIOTIN,[2] 2 µg DAILY	CALCIUM PANTOTHENATE [3] 150 µg DAILY	PANTHENOL [4] 150 µg DAILY	FOLIC ACID,[5] 2 µg DAILY	DESOXY-CORTICO-STERONE-ACETATE [6]
	1	+	+	0	+	0
	2	+	0	+	+	0
	3	0	0	0	0	0
	4	+	0	0	0	0
	5	+	+	0	0	0
Basal diet 1	6	+	0	+	0	0
	7	+	0	0	+	0
	8	0	0	0	0	—[6]
	9	0	0	0	+	0
	10	0	+	0	0	0
	11	0	0	+	0	0
	12	0	+	0	+	0
	13	0	0	+	+	0
	14	0	0	0	0	0
	15	+	0	0	0	0
Basal diet 2	16	0	+	0	0	0
	17	0	0	+	0	0
	18	+	+	0	0	0
	19	+	0	+	0	0
	20	0	0	0	+	0

[1] Each group consisted of 10 animals except groups 3 and 14, which had 16 animals each.

[2] d,l-Biotin, synthetic.

[3] d,l-Calcium pantothenate (crystalline).

[4] d-Panthenol, synthetic (pantothenyl alcohol = alcohol of pantothenic acid).



[5] Folic acid, synthetic.

[6] Synthetic desoxycorticosterone-acetate. In group 8, 4 animals received 0.1 mg, three animals received 0.2 mg, and three animals received 0.3 mg daily.

supplements were as follows: A stock solution was made by dissolving 116.5 mg thiamine hydrochloride, 116.5 mg pyridoxine hydrochloride, 233 mg riboflavin, 5.825 gm nicotinic acid, 23.3 gm choline chloride, 1.456 gm para-aminobenzoic acid, 2.912 gm *i*-inositol, 2.33 gm vitamin E, and 466 mg vitamin K in distilled water at about 40°C. and diluting to 500 ml. One part of this solution was diluted with 4 parts of a 10% sucrose solution and 1 ml of the resultant preparation fed in supplement cups to each rat three times a week. This supplied the amounts indicated in table 1. A similar procedure was followed for the experimental variables listed in table 2. The fat soluble vitamins A and D were provided in the cod liver oil incorporated in the basal diets. Diets were made up in sufficient quantity for a week's feeding. The experimental variables that were added to the two basal diets each week for the 20 groups of rats are shown in table 2.

By the end of the 12th week of the experiment the rats either died or were sacrificed. The tongue, teeth and supporting structures, and viscera were prepared for histological investigation. The results of this part of the study will be reported later.

RESULTS AND DISCUSSION

Gross symptoms

During the course of the experiment the animals were examined for the symptoms which have been previously noted in the rat in pantothenic acid and biotin deficiency. These are listed in table 3. An evaluation of these symptoms based on severity, time of appearance and duration is given in this table.

The rusting of the fur appeared to be a symptom associated with pantothenic acid deficiency. This is shown by the fact that it was prevented by the addition of calcium pantothenate to the diet (groups 10 and 16) and was present in those groups where calcium pantothenate was absent (groups 3, 4, 7, 8, 9). However, the addition of biotin to basal diet 2 seemed to increase the need for pantothenic acid in this regard; i.e., when

TABLE 5

Gross symptoms observed on various diets [1,2]

	Basal diet 1 plus experimental variables as indicated													Basal diet 2 plus experimental variables as indicated						
	Control: Biotin, Ca pantothenate and folic acid	Control: Biotin, panthenol and folic acid	No experimental variables	Biotin	Ca pantothenate and biotin	Biotin and panthenol	Biotin and folic acid	Desoxycorticosterone	Folic acid	Ca pantothenate	Panthenol	Ca pantothenate and folic acid	Panthenol and folic acid	No experimental variables	Biotin	Ca pantothenate	Panthenol	Biotin plus Ca pantothenate	Biotin plus panthenol	Folic acid
Group number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Arched back [3]																1		1		5
Dermatitis [3,4]	1		2	3							1		1	2		2	1	1		
Cropped fur		±	+++	+	±	±	++	+++	+++	±	±	+	+	+++	+	++	++	+	[illegible]	+
Rusting fur		+	+++	+	±	±	++	++	++	±	+	+	+	[illegible]	[illegible]	[illegible]	[illegible]	±	[illegible]	[illegible]
Alopecia body	±		++	++	±	±	+	++	++	±	±	±	±	++	±	++	++	+	±	++
Alopecia eyes			±	±										[illegible]						++
Alopecia mouth			+				±		±					+	[illegible]	++	++	+	±	+
"Spectacle-eyes"			±	±			±	±	±					+	±	++	+	±		±
Caked whiskers			±				±	±	±					+	±	++	++	±		±
Crusting of ears and toes			±	±		±	±	±	±	±	±	±	±	++	±	++	++	±	±	++
Scaling of paws			+	+			+	++	++			±	±	+	±	+	+	±	±	+
Crusting of tail			+	±		±	±	±	±	±	±	±	±	+++	+	+	+	+	±	+++
Decolorization of teeth			±	±		±		±	±	±	±	±	±		±		±	±	±	
Porphyrin nose			+	+			+	+	+			±		±	±	+	+	±		±
Fissures at angles of mouth			++	±			+	+	+	±	±		±	++		+	+	+		+
Deposit on teeth			±	±			±	+	±	±				±	±		±			
Edema of mouth														+						±
Swollen eyelids														+		±	±			

[1] The symptoms noted in this table are not due to an amino acid deficiency since the proteins in both diets are regarded as "complete proteins" at the levels used.

[2] Blank space indicates absence of the specific symptoms. ± = Present but at negligible levels. + = Mild. ++ = Moderate. +++ = Severe.

[3] Numbers refer to number of animals in each group with the respective condition.

[4] Dermatitis consisted of a crusting type of lesion.

neither biotin nor calcium pantothenate was added to basal diet 2 (group 14) rusting did not appear. It did appear when biotin alone (group 15) was added or when biotin and calcium pantothenate (group 18) were added. The "spectacle-eye" condition, caked whiskers, crusting of the ears and tail, edema of the mouth and swollen eye-lids were associated chiefly with the biotin deficient diet, basal diet 2. Porphyrin around the nose was more marked on basal diet 1, while alopecia, scaling of paws and fissures at the angles of the mouth, appeared with equal frequency on both diets.

When calcium pantothenate was added to basal diet 1 (group 10) most, but not all, of the symptoms were alleviated. The addition of panthenol (group 11) produced about the same effects as the addition of calcium pantothenate.

The addition of biotin to basal diet 1 (group 4) led to improvement as regards cropping of the fur, rusting of the fur, crusting of the tail and fissures at the angles of the mouth.

The addition of calcium pantothenate or panthenol, and biotin and folic acid to basal diet 1 (groups 1 and 2) gave the best results. The addition of biotin and folic acid (group 7) was not as effective as biotin alone (group 4). The addition of folic acid (group 9) to basal diet 1 provided no relief.

The animals receiving calcium pantothenate and folic acid (group 12) and the animals receiving panthenol and folic acid (group 13) were not protected as well as those animals receiving calcium pantothenate or panthenol alone (groups 10 and 11).

The addition of biotin to basal diet 2 (group 15) materially alleviated the symptoms but did not offer complete protection. The addition of calcium pantothenate (group 16) or panthenol (group 17) to basal diet 2 was not found to be beneficial. The effect of biotin and calcium pantothenate combined (group 18) did not vary greatly from that seen when biotin alone was added, although the combination of biotin and panthenol (group 19) appeared to be somewhat more effective. Folic acid was not found to be an effective supplement in biotin deficiency.

Sex differences were noted on several diets. The males were more severely affected than the females in group 3, basal diet 1, no supplements; group 7, basal diet 1, with biotin and folic acid added; group 14, basal diet 2, with biotin added; and group 18, basal diet 2, with biotin and calcium pantothenate added. The differences between the sexes, however, were not marked.

Autopsy findings

The mouth symptoms observed at autopsy in groups fed basal diet 2 consisted chiefly of bullous lesions on the tongue. Both clear and hemorrhagic bullae were seen. In some animals a pattern somewhat resembling "geographic tongue" in man was found. The groups affected most severely with tongue symptoms were as follows: group 7, on basal diet 1, with biotin and folic acid added (necrotic lesions); group 14, on basal diet 2, with no experimental variables (bullous lesions); group 16, on basal diet 2, with pantothenic acid added (bullous lesions); and group 20, on basal diet 2, with folic acid added (bullous lesions). A few isolated tongue lesions were found in groups 8 (increased redness), 12 (ulcers) and 17, 18, 19 (blisters).

Weight

Figures 1 and 2 show the weight curves based on the average weights of the animals in each group taken at weekly intervals ($\pm$ 3 days) following the first 4 weeks of the experiments.

Basal diet 1 (group 3) unsupplemented by the dietary variables of interest produced a severe depression in weight which was slightly improved in group 4 (biotin added), while the growth curve was somewhat depressed in the groups that received folic acid (groups 7 and 9) and desoxycorticosterone-acetate (group 8). Both calcium pantothenate (group 10) and panthenol (group 11) proved to be completely satisfactory supplements in this regard.

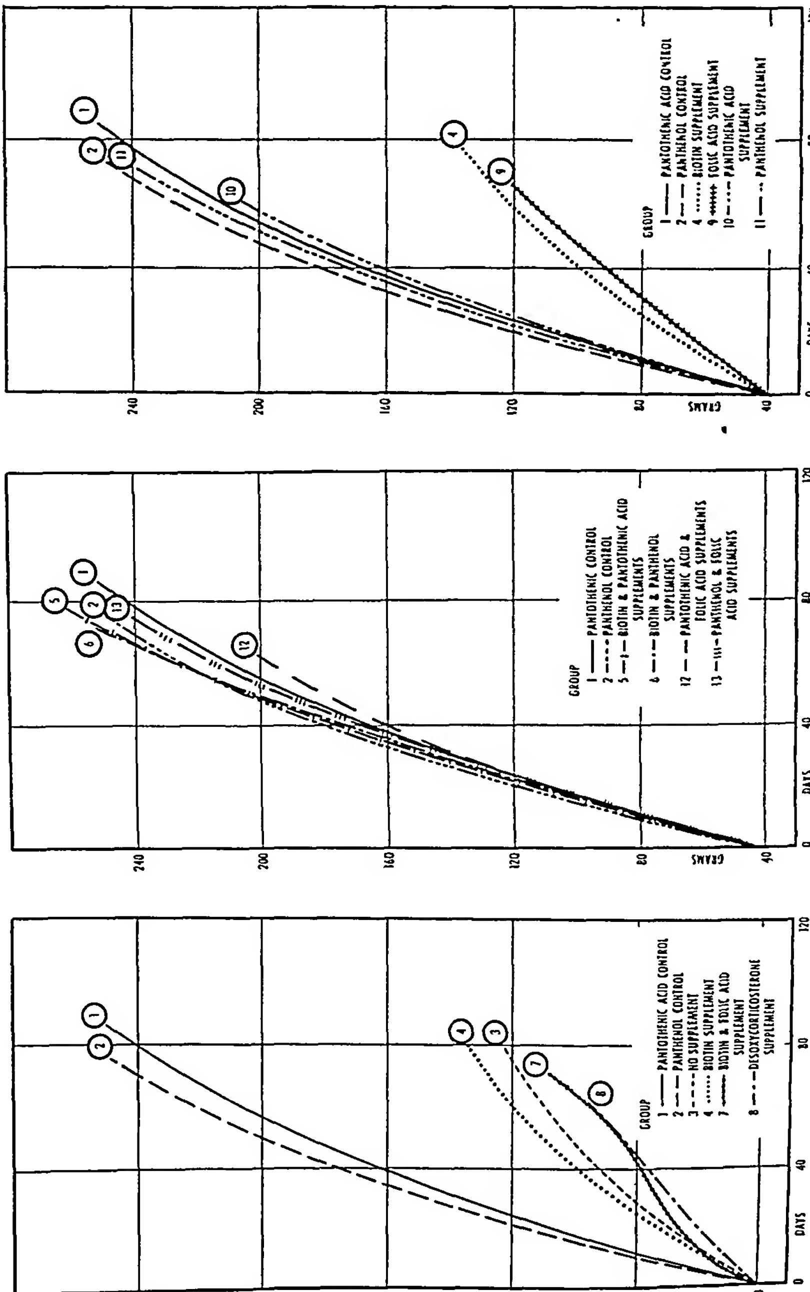


Fig. 1 Growth curves of groups of rats fed diets with or without supplements indicated.

In the biotin deficient animals fed basal diet 2 the addition of biotin (group 15) appeared to provide complete protection, the addition of calcium pantothenate (group 16) or panthenol (group 17) provided slight protection, and again folic acid

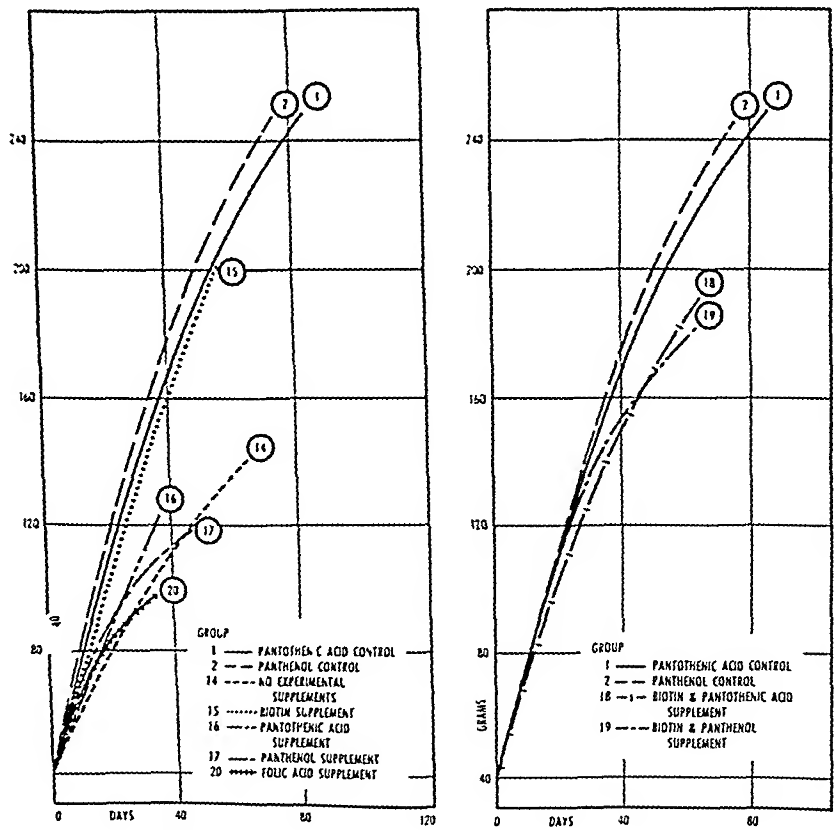


Fig. 2 Growth curves of groups of rats fed diets with or without supplements indicated.

(group 20) depressed the weight curve. The combinations of biotin and calcium pantothenate (group 18) and biotin and panthenol (group 19) were found to have no more effect than biotin alone. The findings as to weight did not exactly parallel those relating to the skin or oral symptoms.

SUMMARY

This investigation is concerned with the changes produced in rats fed one of two synthetic diets complete in every respect except for pantothenic acid, panthenol (a synthetic alcohol of pantothenic acid), biotin and folic acid, which were the experimental variables. Basal diet 2 differed from basal diet 1 in that egg white replaced the casein and dextrin replaced sucrose. With each diet the 4 vitamins were omitted for some groups of animals, added singly for others, and given in combinations to the remaining groups. Those animals receiving basal diet 1 unsupplemented by the experimental variables were chiefly on a pantothenic acid deficient diet, while basal diet 2, unsupplemented, emphasized a lack of biotin. By the end of the 12th experimental week the rats either died or were sacrificed. Skin manifestations and weights were studied.

Unprotected groups subsisting on basal diet 1 showed ulcers of the tongue in various sizes characterized by a granular type of necrosis. Unprotected groups subsisting on basal diet 2 showed grossly clear and hemorrhagic vesicles on the tongue of subepithelial or subdermal origin.

The action of panthenol was in general similar to that of calcium pantothenate under all conditions. Biotin plus folic acid was not as effective as biotin alone when calcium pantothenate or panthenol was absent from the diet. Similarly, calcium pantothenate or panthenol plus folic acid was not as effective as calcium pantothenate or panthenol alone when biotin was absent from the diet.

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LONG-TERM EXPERIMENTS AT OR NEAR THE OPTIMUM LEVEL OF INTAKE OF VITAMIN A [1]

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General acceptance of the principle that with some nutrients there are important differences between the amounts needed for merely or barely adequate, as compared with optimum nutrition, naturally leads to a growing interest in a related concept. When is there simply a *point*, and when is there a *plateau*, of optimum response? And when the latter is the case, is the plateau equally wide with respect to different criteria of nutritional well-being? The data which follow are offered as throwing some light upon these questions as they apply to vitamin A.

Previous work in this Department has shown that when rats of like age, sex, size and genetic and nutritional background are given diets alike in other respects but containing, respectively, three, 6 and 12 I.U. of vitamin A per gram of air-dry food mixture, the increases of intake of this vitamin resulted in increased gains of body weight with decreased coefficients of variation (Sherman and Campbell, '45), as well as larger bodily stores of the vitamin and increased length of life (Caldwell, MacLeod and Sherman, '45; Campbell, Udiljak, Yarmolinsky and Sherman, '45; Sherman, Campbell, Udiljak and Yarmolinsky, '45).

[1] Aided by grants from the Nutrition Foundation, Inc., to Columbia University.

The present paper reports the results of comparisons of the same and other responses to feedings at the two levels of 12 and 24 I.U. of vitamin A per gram of air-dry food, or approximately 3.2 and 6.4 I.U. per Calorie, respectively. As judged by several criteria of nutritional well-being throughout the life cycle, response as a whole is more favorable at 12 I.U. than at either the 6 or the 24 I.U. level of intake of vitamin A. Hence the findings here reported compare the effects of an approximately optimum intake of vitamin A with those of a two-fold higher level of intake.

EXPERIMENTAL

The general methods used were those employed in the other feeding researches published from this Department at intervals since 1921, and the experimental animals were rats from the same long-established, laboratory-bred Osborne-Mendel branch of the Wistar Institute strain. The basal diet was our Laboratory Diet No. 16, often, for convenience, referred to as diet A. It consists of five-sixths ground whole wheat and one-sixth dried whole milk, with table salt 2% of the weight of the wheat, and pure water ad libitum. The extra vitamin A was added in the form of high grade cod liver oil, of such potency that the amount of food mixture it displaced was negligible, averaging only about one part in 400. As previous experiments have consistently shown that animals bred, housed, handled and fed as were those here used do not respond to added vitamin D, this was deemed adequate evidence that their supplies of vitamin D are such that the benefit resulting from added cod liver oil is attributable to its vitamin A. While obviously it is conceivable that some unknown constituent still present in highly refined cod liver oil may be a significant factor contributing to these results, there is no positive indication of any such substance. Diet 361 had 12 I.U. and diet 362 had 24 I.U. of vitamin A per gram of air-dry food. Both averaged 14.2 to 14.4% of protein.

The rats were weighed weekly throughout their lives, and the pregnant females were weighed every second day.

The air-dry food mixture was always available to the animals and records of their food consumption were kept from the age of 28 days throughout the lives of the first generation and from the 28th day to the 56th day of life in the second. Of diets 361 and 362 containing respectively 12 and 24 I.U. per gram of air-dry food, the rats ate essentially the same amounts: 0.49 to 0.42 Cal. per gram of body weight per day at 5 to 7 weeks of age; 0.35 to 0.25 at two to three months; 0.23 to 0.21 at 4 to 6 months; and about 0.20 at 6 months and over. Early growth was very slightly more efficient, both per 1,000 Cal. and per gram of protein consumed, on the diet having 12 than on that having 24 I.U. per gram.

The experimental animals were divided into matched lots so that those receiving one of the two diets which were being compared were exactly duplicated by those receiving the other diet. Each experimental lot consisted of three females and two males. As each female showed pregnancy she was separated from the group and kept in a cage by herself until her young were 4 weeks old; then she was returned to her original cage. The offspring were used in part to make up second generation breeding lots and in part for the studies of body storage reported below.

Growth, breeding records, and length of life

The data on growth, breeding records, and length of life yielded by strictly comparable lots of animals fed, respectively, diet 361 with 12 I.U. and diet 362 with 24 I.U. of vitamin A per gram are summarized in table 1.

Storage of vitamin A in the liver

Having previously studied bodily storage at ages up to 300 days (Campbell, Udiljak, Yarmolinsky and Sherman, '45), the experiments here reported show the amounts of vitamin A found in the livers of rats of both sexes at the ages of 500 and of 700 days. As in the work of the above authors, the determinations were made by the single feeding method described

TABLE 1

Growth, weight, breeding records, and length of life of rats on two diets differing in vitamin A content

	ON DIET 361 WITH 12 I.U./GM			ON DIET 362 24 I.U./GM		
	No. of cases	Mean with its P.E. [1]	C.V. [2]	No. of cases	Mean with its P.E. [1]	C.V. [2]
Gain in wt., 28th to 56th day of life						
Males (gm)	24	65.6 ± 0.71	8	24	65.9 ± 1.02	11
Females (gm)	36	54.3 ± 0.62	10	36	54.6 ± 0.56	9
Wt. at 84 days of age						
Males (gm)	24	184.0 ± 1.95	8	24	184.7 ± 1.68	7
Females (gm)	26	151.3 ± 1.40	7	29	152.1 ± 1.30	7
Wt. at 100 days						
Males (gm)	24	217.3 ± 1.93	7	24	213.3 ± 2.25	8
Females (gm)	25	167.3 ± 1.55	7	28	170.8 ± 1.69	8
Wt. at 120 days						
Males (gm)	24	248.3 ± 2.00	6	24	240.3 ± 2.86	9
Females (gm)	33	179.8 ± 1.63	8	33	180.3 ± 1.79	9
Wt. at 150 days						
Males (gm)	24	277.5 ± 2.17	6	24	269.7 ± 3.03	8
Females (gm)	25	186.6 ± 1.61	6	32	190.6 ± 1.49	7
Wt. at 180 days						
Males (gm)	24	298.5 ± 2.63	6	24	290.6 ± 3.05	8
Females (gm)	31	198.5 ± 1.86	8	33	201.0 ± 2.13	9
Wt. at 210 days						
Males (gm)	24	313.5 ± 3.21	8	24	302.0 ± 2.81	7
Females (gm)	27	205.2 ± 1.85	7	32	208.9 ± 1.99	8
Wt. at 240 days						
Males (gm)	24	323.0 ± 3.87	9	23	309.6 ± 2.89	7
Females (gm)	33	205.8 ± 1.65	7	32	211.1 ± 2.04	8
Wt. at 300 days						
Males (gm)	24	329.4 ± 4.08	9	22	320.5 ± 3.16	7
Females (gm)	30	218.4 ± 1.95	7	32	216.2 ± 1.81	7
Wt. at 365 days						
Males (gm)	24	333.5 ± 4.21	9	22	318.5 ± 3.21	7
Females (gm)	27	220.7 ± 2.62	9	35	220.6 ± 1.93	8

TABLE 1 (*continued*)

	ON DIET 361 WITH 12 I.U./GM			ON DIET 362 24 I.U./GM		
	No. of cases	Mean with its P.E.[1]	C.V.[2]	No. of cases	Mean with its P.E.[1]	C.V.[2]
Wt. at 500 days						
Males (gm)	21	311.0 ± 6.57	16	19	305.7 ± 4.78	10
Females (gm)	29	231.1 ± 2.35	8	31	231.9 ± 2.74	10
Wt. at 600 days						
Males (gm)	17	310.6 ± 8.29	16	15	287.3 ± 5.85	12
Females (gm)	30	236.0 ± 2.28	8	27	228.3 ± 2.73	9
Wt. at 700 days						
Males (gm)	14	291.0 ± 8.49	16	8	268.0 ± 10.95	17
Females (gm)	26	225.6 ± 2.63	9	25	217.0 ± 3.42	12
Age at birth of first young (days)	36	112.2 ± 2.84	23	35	112.6 ± 3.25	26
Number of young borne per female	36	42.3 ± 1.63	35	36	32.3 ± 2.04	57
Number reared	36	31.6 ± 1.73	49	36	24.4 ± 1.80	66
Reproductive life of females (days)	36	386.9 ± 15.16	35	36	292.3 ± 19.09	59
Length of life						
Males (days)	24	715.9 ± 14.93	15	24	646.7 ± 20.54	23
Females (days)	36	794.1 ± 18.01	20	36	777.0 ± 18.27	21

[1] P.E. = the "classic" probable error of the mean.
[2] C. V. = coefficient of variation.

by Sherman and Todhunter ('34). The sexes were kept separate in these studies. The results are shown in table 2.

DISCUSSION

In the experiments here reported the rates of early growth and their variability were consistent with those found by Sherman and Campbell ('45). With the males, but not the females, the highest intake level was associated with a very slightly less rapid early growth.

In later growth the females showed practically the same results on the two intake levels here studied, while the males grew very slightly less on the higher level. Coefficients of variation were low for both levels and both sexes.

Increasing the vitamin A content of the diet from 12 to 24 I.U. per gram appears to have shortened the length of life. A much larger proportion of males died under the age of two years on the intake level of 24 I.U. than on that of 12 I.U.,

TABLE 2

Vitamin A in I.U. per gram of rat liver (fresh weight)

	MALES			FEMALES		
	No. of cases	Mean with its P.E. [1]	C.V. [2]	No. of cases	Mean with its P.E. [1]	C.V. [2]
At 500 days on:						
Diet 16 (3 I.U.)	9	6 ± 1.3	101	5	2 ± 0.6	105
Diet 360 (6 I.U.)	10	71 ± 6.4	43	16	165 ± 6.0	22
Diet 361 (12 I.U.)	19	771 ± 24.6	21	21	827 ± 40.6	34
Diet 362 (24 I. U.)	10	1453 ± 80.9	27	10	1485 ± 49.4	16
At 700 days on:						
Diet 361 (12 I.U.)	6	1333 ± 141.3	39	11	1373 ± 93.9	34
Diet 362 (24 I.U.)	3	2700 ± 88.6	9	9	3106 ± 122.5	18

[1] P.E. = the "classic" probable error of the mean.
[2] C.V. = coefficient of variation.

which appears to be optimum. With the females this difference was not as great. Females on the two levels bore their first young at the same age, but the reproduction record as a whole was less variable and averaged distinctly better on the (presumably optimum) level of 12 I.U. than on the two-fold higher level of 24 I.U. per gram of dry food.

While based upon differently determined criteria of minimum adequacy, our findings as to the vitamin A requirements

(minimum and optimum) of the rat are in general agreement with those of Goss and Guilbert ('39), of Lewis, Bodansky, Falk and McGuire ('41) and of Paul and Paul ('46). The highest concentrations of vitamin A in fresh liver tissue found for rats in this study are about one-fifth those found for bears and seals by Rodahl and Moore ('43).

On the diet containing 3 I.U. of vitamin A per gram of air-dry food, body stores were negligible. On intake levels of 6, 12, and 24 I.U. per gram of food, the body stores of vitamin A increased progressively with each increase of the intake level. Experiments at the 12 and 24 I.U. levels showed that body stores were higher at 700 days than at 500 days of age. These results were true of both sexes.

SUMMARY

In experiments with rats, an increase of vitamin A in the diet from 12 I.U. to 24 I.U. per gram did not measurably influence growth between the ages of 28 and 56 days. This was also true of later growth and adult size in the females. Males grew to slightly lower adult weights on the diet with 24 than on that with 12 I.U. per gram of food.

The ages of the females at the birth of their first young averaged the same for these two diets. The average weights of the young at 28 days of age were also the same.

In length of reproductive life, number of young borne, number of young reared, percentage reared, and total weight of young at 28 days, higher records were made by the females on diet 361 than by those on diet 362; that is, these values were more favorable with 12 I.U. than with 24 I.U. of vitamin A per gram of food. This was also true of length of life (appreciably for males, but only very slightly for females). Whether these small differences are more than accidental we do not know; their chief significance is in showing that nutritional response is essentially "plateaued" at this intake level.

As in all comparable work in this laboratory, the females lived longer than the corresponding males. By comparing the data of this with those of our previous papers, already noted,

it is clearly shown that quadrupling an adequate (or minimum adequate) allowance of vitamin A may increase the normal length of life by 10 to 12%; the "useful" segment of the life cycle — from the attainment of maturity to the onset of senility — is increased also and in greater ratio. Thus it is shown that such an increase in intake of vitamin A, above the minimum adequate level, expedites development and defers old age in the same individuals. In these experiments the optimum concentration of vitamin A in the diet was at or somewhat above 12 I.U. per gram of air-dry food, or about 4 times the level usually accepted as minimum adequate.

Storage of vitamin A in the body of the rat increased with the intake level (from 3 I.U. up to 24 I.U. per gram of dry food) in both sexes and was greater at 700 than at 500 days of age, indicating that the ability to store this vitamin continues throughout middle age.

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THE EFFECTS OF VARIOUS LEVELS OF THIAMINE AND RIBOFLAVIN INTAKE UPON THE UTILIZATION OF CASEIN, SUPPLEMENTED WITH METHIONINE [1]

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The literature concerning the effects of vitamin deficiencies upon nitrogen metabolism provides some evidence of a general physiological relationship between the vitamins and the amino acids. Some of these reports indicate that subopitimum levels, or the absence, of either thiamine or riboflavin may adversely affect the digestibility and biological value of the ingested protein (Basu and Nath, '42; Kon, '31; Sure, '41; Borgstrom and Hammersten, '44; Richter and Rice, '44; and Diaz et al., '47). Furthermore, Sure and Ford ('42) found an interrelationship between thiamine and riboflavin indicating that the efficiency of riboflavin utilization was decreased in the absence of thiamine, but that the reverse was not true. The study reported herein presents data on the effects on the digestibility and biological value of casein, supplemented with methionine, of varying the levels of both thiamine and riboflavin.

EXPERIMENTAL

The nitrogen balance technique (Mitchell, '24; Mitchell and Carman, '26; Mitchell and Hamilton, '44) was used with minor modifications to measure the digestibility and bi-

[1] Contribution from Montana State College, Agricultural Experiment Station Paper No. 214, Journal Series.

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ological value of the casein. Young male rats of the Sprague-Dawley strain were used as the test animals. They were taken from litters whose mothers had received a commercial dog food [3] until the 16th day of lactation, when they were given a diet low in thiamine and riboflavin, diet I (see table 1 for composition of test diets). The weanling males (35 to 40 gm) were randomized, placed in individual metabolism cages, maintained in a room at a temperature of 78–80°F. and fed a purified diet (diet II) free of thiamine and riboflavin. Some of the rats were given a large daily supplement of thiamine (50 μg) and thus depleted only of riboflavin. Similarly, others were given 50 μg of riboflavin and depleted of thiamine. When their weights were constant for three days the rats were considered depleted of their major vitamin reserves. This occurred in about 10 to 12 days when thiamine was absent from the diet, in about 7 days when riboflavin was omitted, and in about 10 to 12 days when both vitamins were withheld. They were then fed diet III, also free of thiamine and riboflavin but containing a lower amount of protein, about 10%. In diets II, III and IV dextrose was substituted for cornstarch since it has been shown that there is less intestinal synthesis of the B-complex vitamins when dextrose is the source of carbohydrate (Guerrant and Dutcher, '34; Guerrant et al., '37; Griffith, '35; Morgan et al., '38; and Mannering et al., '44).

Mitchell's technique was modified and all groups of rats, with the exception of group 2, received diet III and the same supplement in both the 7-day experimental periods, A and C, and a protein-free ration, diet IV, in the 7-day standardizing period, B. It was necessary to do this because the usual standardizing diet containing 4% egg protein would provide appreciable amounts of the vitamins under investigation. Rats receiving the higher levels of the vitamins were given the same amount of basal diet as were those on the lower levels. The total food eaten per rat per period was recorded (see table 2). Ferric oxide was added to the food as a marker on the first and last day of each test period. Feces for one entire period were

[3] Purina dog chow.

placed in concentrated sulfuric acid in a Kjeldahl flask, digested, made to volume and sampled.

A few of the rats, used as controls, were not depleted of either vitamin and received the commercial dog food up to the time they were put on the experiment. Some of these con-

TABLE 1

Composition of experimental diets

CONSTITUENT		DIET					
		I	II	III	IV	V	VI
		gm	*gm*	*gm*	*gm*	*gm*	*gm*
Cornstarch		36				61	66.6
Dextrose		15	63	71	81	10	10
Casein, purified [1]			18	9.4		9.4	
Casein, crude		30					
DL-Methionine [1]				0.6		0.6	
Egg, whole dried							4.4
Cottonseed oil		11	11	11	11	11	11
Mineral mixture [2]		4	4	4	4	4	4
Agar		2	2	2	2	2	2
Sodium chloride		1	1	1	1	1	1
Fish liver oil [3]		1	1	1	1	1	1
Vitamins:							
Alpha-tocopherol	mg		2.24	2.24	2.24	2.24	2.24
Ascorbic acid	mg		0.5	0.5	0.5		
Biotin (free acid)	µg		2.25	2.25	2.25		
Choline chloride	gm	0.15	0.25	0.25	0.25	0.15	0.15
Folic acid	µg		0.06	0.06	0.06		
Inositol	gm		0.10	0.10	0.10		
Nicotinic acid	mg		0.63	0.63	0.63		
Calcium pantothenate	mg	0.30	0.50	0.50	0.50	0.30	0.30
p-Aminobenzoic acid	mg		0.30	0.30	0.30		
Pyridoxine hydrochloride	mg	0.30	0.63	0.63	0.63	0.30	0.30
Vitamin K	mg		0.21	0.21	0.21		
Riboflavin	mg					0.40	0.40
Thiamine hydrochloride	mg					0.50	0.50

[1] Vitamin test casein (14.9% N; 0.14 µg riboflavin per gram; 0.0 µg thiamine) and DL-methionine, GBI, manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Mitchell ('44) mineral mixture No. 446.

[3] Three thousand U.S.P. XI units of vitamin A and 400 A.O.A.C. units of vitamin D per gram.

TABLE 2

The effect of various levels of thiamine and riboflavin intake upon the utilization of casein, supplemented with methionine, in diet III

GROUP NO.	VITAMIN SUPPLEMENT: Thiamine HCl	VITAMIN SUPPLEMENT: Riboflavin	PERIOD	AVERAGE PER PERIOD[1]: Food eaten per rat	AVERAGE PER PERIOD[1]: Weight of rat	AVERAGE PER PERIOD[1]: Gain in weight of rat	AVERAGE PER PERIOD[1]: Digestibility	AVERAGE PER PERIOD[1]: Biological value	AVERAGE BIOLOGICAL VALUE[2] (both periods)
	μg/day	*μg/day*		*gm*	*gm*	*gm*	%	%	%
	No depletion								
1	50	50	A	37	73	6	100	87	
			C	36	79	7	99	91	89
2	Diet V Mitchell		A	55	84	12	100	92	
			C	54	119	13	100	97	95
	Thiamine depleted								
3	0.00	50	A	24				.	
			C					.	..
4	1.25	50	A	26	62	0	100	46	
			C	29	55	0	99	61	53[3]
5	2.50	50	A	31	66	1	99	69	
			C	33	69	5	99	82	76
6	5	50	A	38	69	6	99	88	
			C	37	74	7	97	88	88
7	10	50	A	39	74	6	99	85	
			C	39	78	8	98	89	87
8	20	50	A	39	72	9	100	92	
			C	37	78	9	100	97	95
9	50	50	A	39	74	7	99	88	
			C	37	78	8	97	91	90
	Riboflavin depleted								
10	50	0	A	31	53	2	99	71	
			C	28	50	1	97	44	58[4]
11	50	1.25	A	34	63	3	100	81	
			C	32	64	3	98	81	81
12	50	2.50	A	34	55	5	99	80	
			C	33	61	4	98	82	81
13	50	5	A	35	61	6	99	90	
			C	35	68	8	98	92	91–
14	50	10	A	35	64	8	100	87	
			C	35	73	8	98	90	89
15	50	20	A	35	65	8	100	96	
			C	35	72	7	98	94	95
16	50	50	A	35	61	8	99	94	
			C	35	69	8	99	94	94
	Thiamine and riboflavin depleted								
17	2.50	1.25	A	34	62	5	99	65	
			C	30	63	2	97	81	73
18	2.50	2.50	A	31	56	5	100	73	
			C	29	60	4	98	85	79
19	5	1.25	A	34	58	4	100	74	
			C	30	61	3	97	80	77
20	5	2.50	A	35	60	7	100	80	
			C	32	66	5	98	87	83

[1] Average of 4 replicates.
[2] Average of 8 determinations.
[3] Difference in means necessary for significance at a 5% level is 11.7%. See table 3.
[4] Difference in means necessary for significance at a 5% level is 10.8%. See table 4.

trol rats were placed in *group 1* and fed the maximum levels of both of the vitamins. Others, placed in *group 2*, received diet V in periods A and C and diet VI in period B. The latter were treated according to the specific directions of Mitchell and Hamilton ('44) in order to determine, under standard conditions, the biological value of the casein, supplemented with methionine. Purified diets V and VI were those proposed by Mitchell and Hamilton ('44).

Other rats depleted of thiamine were placed in *groups 3 to 9* and received one of the following thiamine hydrochloride supplements in micrograms: 0.0, 1.25, 2.5, 5.0, 10.0, 20.0, 50.0, together with 50 µg of riboflavin per day. The riboflavin-depleted rats were placed in *groups 10 to 16* and given similar levels of riboflavin with 50 µg of thiamine.

The vitamin mixture added to diets II, III and IV, table 1, was based on levels suggested by Boutwell et al. ('45) and contained 7 vitamins not included in the regular Mitchell diet. The thiamine and riboflavin supplements were in solution and this was added daily to the food.

Since facilities were inadequate, it was impossible to carry on the desirable number of replications at one time. However, a complete replicate of groups 1 through 16 was made during each of 4 experimental periods. There were two test periods in a replicate and hence 8 figures for biological value were obtained for each level of the two vitamins.

To determine whether intestinal synthesis of riboflavin was taking place, as suggested by Ziegler ('46), 2% sulfasuxidine[4] (succinylsulfathiazole, Welch and Wright, '43) was included in diets III and IV at the expense of the dextrose, and a complete replicate of the riboflavin levels was carried out. It will be noted that diets III and IV contained known amounts of para-aminobenzoic acid, biotin, folic acid and methionine — factors which Welch and Wright ('43) found effective in combatting the growth-inhibiting effect of another sulfa compound, sulfaguanidine. Since the results indicated that the sulfasuxidine caused no marked difference in growth or in biological

[4] Sulfasuxidine donated by Sharp and Dohme, Inc., Glenolden, Pa.

value, it seemed probable that intestinal synthesis of riboflavin was not taking place on this diet, containing dextrose as the carbohydrate, and the data relating to it are therefore not presented in this paper.

On the basis of the results of the first series, in which the requirement of thiamine or riboflavin was studied in the presence of adequate amounts of the other vitamin, a second series was devised in which lower amounts of each of the vitamins were used. Since the rats in the first series receiving 1.25 μg of thiamine failed to grow and developed *polyneuritis, this level was not repeated; thiamine was fed at* either 2.5 or 5.0 μg and riboflavin at either 1.25 or 2.50 μg. The 4 possible combinations of these levels were fed to rats that had been depleted of both thiamine and riboflavin, *groups 17 to 20*, table 2.

Seven-day fecal and urinary collections were made after a 5-day stabilization period, and nitrogen determinations on the excretions and diets followed the Kjeldahl method. Values for endogenous nitrogen for each of the experimental periods A and C were calculated by correcting the urinary nitrogen of period B for body weight and metabolic nitrogen by adjusting the fecal nitrogen of period B for food consumption.

The biological values and digestibilities were calculated by the following formulae:

$$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100 = \text{Biological Value;}$$

$$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100 = \text{Percentage Digestibility.}$$

RESULTS AND DISCUSSION

Data showing the effects of various levels of thiamine and riboflavin intake on the digestibility and biological value of casein, supplemented with methionine, are summarized in table 2. It is obvious that the level of thiamine and riboflavin intake did not affect the digestibility of the test protein, as all values were between 97 and 100%.

The average biological values of the test protein ranged from 53 to 95%, and these findings, though limited in number,

were treated statistically according to the methods of Snedecor ('46) and Paterson ('39). The analysis of variance of the biological values, together with the difference in means necessary

TABLE 3

Analysis of variance for data obtained on the biological value of casein, supplemented with methionine, in groups 3 to 9

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F. VALUE
Levels of thiamine	5	1810.2	13.5 [1]
Replications	3	42.2	
Error	39	134.6	
Total	47		

[1] Total exceeds the 1% point for the distribution of F. (Snedecor, '46). Standard error of difference between means $= \sqrt{\frac{134.6 \times 2}{8}} = 5.8$.

Difference in means necessary for significance =

5.8 × 2.70 (0.01 value of t) = 15.7 at 1% level.

5.8 × 2.02 (0.05 value of t) = 11.7 at 5% level.

TABLE 4

Analysis of variance for data obtained on the biological value of casein, supplemented with methionine, for groups 10 to 16

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN SQUARE	F. VALUE
Levels of riboflavin	6	1336.1	11.7 [1]
Replications	3	145.0	1.3
Error	46	114.6	
Total	55		

[1] Exceeds the 1% point for the distribution of F. (Snedecor, '46). Standard error of differences between means $= \sqrt{\frac{114.6 \times 2}{8}} = 5.35$.

Difference in means necessary for significance =

5.35 × 2.69 (0.01 value of t) = 14.4 at 1% level.

5.35 × 2.01 (0.05 value of 5) = 10.8 at 5% level.

for significance for groups 3 to 9 and 10 to 16, are presented in tables 3 and 4 respectively.

The average biological values for most groups are slightly lower in period A than in the second period, C. Discussion of

this difference has been reviewed by the authors in another paper in this issue (Mayfield and Hedrick, '49), and has received further attention in a recent article by Allison et al. ('46).

Effect of various levels of thiamine intake upon protein utilization

The biological value of the test protein was significantly affected by the level of thiamine intake, as is shown in table 3. Further analysis of the data shows that the difference in means necessary for significance at the 1% level is 15.7 and at the 5% level is 11.7%. Using this per cent as the difference in means necessary for significance, it will be noted that changing Mitchell's basal diets V and VI, containing cornstarch, to diets III and IV, containing dextrose as the major source of carbohydrate, caused no lowering in the utilization of the test protein, since the values of 89 and 95, for control groups 1 and 2, are not significantly different (see discussion on the composition of the experimental diets). Depleting the rats of their body stores of thiamine did not affect this value, since group 9, thiamine depleted, gave a value of 90 as compared to 89 for group 1 with no depletion.

Values representing the utilization of the protein could not be determined for rats continued on a thiamine-free diet, group 3, since the animals did not survive the test periods. The two lower levels of thiamine, 1.25 and 2.50 µg per day, groups 4 and 5, permitted survival, and the biological values were lowered significantly to 53 and 76%, respectively. Rats in group 4, receiving 1.25 µg of thiamine, failed to grow and were the only ones to develop polyneuritis. The feeding of the next 4 supplements at the levels of 5, 10, 20, and 50 µg produced no significant changes, the results being 88, 87, 95 and 90%, respectively.

Changes in body weight of the rats during the experimental periods A and C, expressed as "average gain in weight," are given in table 2. Rats in group 4, receiving 1.25 µg of thiamine per day, were the only animals that did not make some gain in

weight during the test period. Hence there is no indication that the low biological values obtained in groups 4 and 5 were due to a loss of body protein.

Rats receiving 5 µg of thiamine per day, group 6, utilized their protein as efficiently as did those receiving the higher levels. Taking into consideration the amount of food consumed by the rats in this group, the daily supplement of 5 µg of thiamine was comparable to a diet containing 100 µg per 100 gm of food. It is interesting to note that this level, at which the rats made maximum utilization of the test protein, is similar to that reported by Arnold and Elvehjem ('38) for normal growth, i.e., 80 to 100 µg of thiamine hydrochloride per 100 gm of diet.

Effect of various levels of riboflavin intake upon protein utilization

The statistical treatment of the data for groups 10 to 16, as given in table 4, indicates that the biological value of the test protein was significantly affected by the level of riboflavin intake. The difference in means necessary for significance at a 1% level is 14.4 and at a 5% level is 10.8%.

Rats in group 10, table 2, depleted of riboflavin and continued on a riboflavin-free diet, lived through both experimental periods and yielded data indicating an average biological value of 58%. Since in most cases the biological values are slightly lower in period A than in C (as previously discussed), it is probably significant that as the rats in group 10 were further depleted of riboflavin in period C, they were less able to utilize their protein and a low value of 44% was obtained as compared to 71% in period A.

Using 10.8% as the difference in means necessary for significance, table 4, it will be noted that the average biological value of 81% for groups 11 and 12, receiving 1.25 and 2.50 µg of riboflavin per day, was significantly lower than that obtained when the rats received the higher levels of the vitamin. Daily supplements of 5.0 µg of riboflavin allowed the rats to utilize the test protein as efficiently (biological value of 91%)

as did the higher levels of 10, 20 and 50 μg (biological values of 89, 95 and 94%). These values are similar to those obtained with the control groups.

All rats in groups 10 to 16, table 2, showed some gain in weight during the experimental periods and, as discussed in the previous section, there is no indication that the low biological values obtained in groups 10, 11 and 12 were due to a loss of body protein.

These data suggest that a daily supplement of 5 μg of riboflavin was necessary for the maximum utilization of the test protein. This value is similar to that found for thiamine and is comparable to a diet containing 100 μg of vitamin per 100 gm.

Effect of various levels of thiamine and riboflavin intake upon protein utilization

Rats in groups 17 to 20, table 2, depleted of both thiamine and riboflavin, were given the various combination supplements of 1.25 and 2.50 μg of riboflavin and 2.50 and 5.0 μg of thiamine. When the biological value for group 17 is compared with those for corresponding groups 5 and 11, 18 with 5 and 12, 19 with 6 and 11, and 20 with 6 and 12, it will be noted that the values are rather similar and that low levels of both of the vitamins do not seem to have an additive detrimental influence on the utilization of the protein.

SUMMARY

1. The effect of various levels of thiamine and riboflavin intake upon the utilization of casein, supplemented with methionine, was studied. The nitrogen balance technique with weanling rats was used in this investigation, following the general method of Mitchell.

2. Necessary minor modifications in method did not affect the biological value of the test protein: (a) Utilization of the protein was similar when rats received dextrose in place of cornstarch as the source of carbohydrate; (b) depleting the rats of their major reserves of either thiamine or riboflavin

did not permanently affect their ability to utilize the test protein when they were given the various vitamin supplements during the experimental period.

3. The level of thiamine and riboflavin intake did not affect the digestibility of the protein. All digestibility values were between 97 and 100%.

4. Statistical analysis of the data showed that the biological value of the test protein was significantly affected by the level of thiamine or riboflavin intake.

5. Biological values for the test protein, obtained with the control groups of rats, were 89 and 95%.

6. Rats receiving daily supplements of 1.25 and 2.50 μg of thiamine hydrochloride, with all other factors optimum, were unable to utilize the protein as efficiently as did those receiving the larger amounts, and biological values were significantly lowered to 53 and 76%, respectively. Rats receiving 5.0 μg of thiamine daily utilized their protein (biological value of 88%) as efficiently as did those receiving 10, 20 and 50 μg of the vitamin (biological values of 87, 95 and 90%).

7. Groups of rats receiving daily supplements of 0, 1.25 and 2.50 μg of riboflavin did not utilize their protein as efficiently (biological values of 58, 81 and 81%) as did the control groups. At a 5.0 μg level of intake (biological value of 91%), protein utilization was as efficient as at the higher levels of 10, 20 and 50 μg of riboflavin (biological values of 87, 95 and 90%).

8. Feeding supplements of both thiamine and riboflavin at a low level did not show an additive detrimental influence on the utilization of the protein.

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THE EFFECT OF CANNING, ROASTING AND CORNING ON THE BIOLOGICAL VALUE OF THE PROTEINS OF WESTERN BEEF, FINISHED ON EITHER GRASS OR GRAIN[1]

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INTRODUCTION

Interest has developed recently in the nutritive value of protein in foods and how it may be affected by the various processing procedures. The efficiency with which a given protein supplies the nitrogen requirements of an animal is defined in terms of its biological value. An effective protein is one which is highly digestible and absorbable and which supplies the organism with adequate amounts of the amino acids which it needs. Roasting and canning may affect the physical properties of meat protein adversely by changing the linkages so that they are not as susceptible to enzymatic digestion (Hawk et al., '47). Morgan and Kern ('34) found that the biological value decreased in proportion to increase in severity of the heat treatment. The salts used in the corning process, due to their peptizing behavior, may, in contrast, increase the effect of the digestive enzymes by rendering the protein more soluble (Gortner et al., '28). However, due to the long immersion of

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the meat in the concentrated brine, unfavorable physical changes in the proteins may take place and thus decrease their digestibility.

Meat from grain-fed animals is, according to general belief, more tender and palatable than meat from grass-fed animals. Since tenderness may be correlated with digestibility, this investigation was undertaken to study, first, the effect of varying the types of feed, and second, the subsequent changes produced by canning, corning and roasting on the digestibility and biological value of beef protein.

METHODS

The meat was obtained from 17 two-year-old Hereford steers that had been on controlled feeding experiments conducted by the Department of Animal Industry. Three of them were finished on Montana grass, while groups of two, 5 and three animals were fed a barley and beet-pulp combination for the last 60, 90 and 180 days, respectively. Four steers were fed the grain mixture throughout the experimental period. After slaughtering, the meat was allowed to age for approximately 10 days in a dark room at 33–35°F.

Raw beef

The eye muscles of the 12th rib cuts were ground, dehydrated at 60°C., reground, fat-extracted and used in the experimental diets as a control for the experiment on roasted beef (Mitchell and Beadles, '37). Similarly, representative samples of raw beef round were removed from the meat to be used for canning and corning experiments.

Roasted beef

The right and left 10th-to-11th standing-rib cuts from each of the three grass-fed animals were roasted in an open pan at 163°C. (325°F.) to an internal temperature of 80°C. The outer portions were discarded and meat from the eye muscles of the cooked roasts was thoroughly mixed, ground and prepared in the same manner as the raw beef.

Canned beef

Lean beef round was cut into pieces, browned in an open pan in the oven for 30 minutes, packed hot into pint glass jars and processed at 250° F. for 85 minutes, following the recommendation of Stanley et al., of the U. S. Department of Agriculture ('42). The entire contents of the jars were ground and prepared as were the other meats.

Corned beef

Large pieces of beef round were cured for 60 days in a brine consisting of: 2 gal. water, 4 lb. NaCl, 1.5 lb. sugar, 2 oz. $NaHCO_3$, and 1 oz. $NaNO_3$. Representative samples of the corned beef, free from excess brine, were prepared in the same manner as the other meat samples. Compensation was made for the salt content of this meat when it was used in the experimental diet.

Method for determining biological value of protein

Weanling, male albino rats of the Wistar strain were used in the paired-feeding experiments according to Mitchell's nitrogen balance method (Mitchell, '24; Mitchell and Carman, '26; and Mitchell and Hamilton, '44). Each pair of litter mates was used to test the biological value of both the raw and processed samples of meat. One rat of the pair was given the raw sample in the first test period and the processed sample in the second. Four or 5 pairs composed each test group and the paired-feeding technique was used. The percentage composition of the experimental diets was as follows: Sucrose, 10; agar agar, 2; cottonseed oil, 8.5; cod liver oil, 1.5; salt mixture, 4 (Mitchell and Hamilton, '44); protein, 10 to 12 (supplied in ground, dried beef); vitamin supplement, 5 (a mixture of synthetic vitamins with starch); and enough cornstarch to make 100%. The vitamin supplement consisted, in grams, of the following: Calcium pantothenate, 0.03; choline chloride, 15.0; pyridoxine hydrochloride, 0.03; riboflavin, 0.04; thiamine hydrochloride, 0.05; these vitamins, in the amounts indicated,

were mixed with 484.85 gm of cornstarch. When the daily protein intake of the rats had been constant for 5 days, the 7-day period (A) was begun. This was followed by a period of low nitrogen intake (B), using a diet containing 4% egg protein, then by another test period (C) with the meat samples of period A reversed. Throughout these three periods the urine and feces were collected daily. Food consumption was controlled and recorded.

Nitrogen analyses of the food and excreta were made by the Kjeldahl method. Since the wastages of dietary nitrogen in digestion are contained in the fecal nitrogen, metabolic nitrogen for periods A or C is calculated by adjusting the fecal nitrogen of period B (standardizing period) for food consumed; e.g., Metabolic Nitrogen = Fecal Nitrogen (period B) $\times \frac{\text{food consumed in period A}}{\text{food consumed in period B}}$. Similarly, the endogenous nitrogen for periods A or C is determined by correcting the urinary nitrogen of period B for body weight; e.g., Endogenous Nitrogen = Urinary Nitrogen (period B) $\times \frac{\text{body weight in period A}}{\text{body weight in period B}}$ (Mitchell, '43). The biological value and digestibility were calculated from the following formulae:

$\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N}) - (\text{Urinary N} - \text{Endogenous N})}{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})} \times 100 =$ Biological value. $\frac{\text{Food N} - (\text{Fecal N} - \text{Metabolic N})}{\text{Food N}} \times 100 =$ Percentage Digestibility.

Analysis of results

The data were treated statistically according to the method of analysis of variance (Snedecor, '46).

RESULTS AND DISCUSSION

In table 1 individual metabolic data are given for 10 rats receiving raw beef round, in order to show the variations obtained in this type of experiment. The remainder of the table is composed of the averages of 4 to 5 rats for each of two periods. Data are presented showing the effects of canning, roasting and corning on the digestibility and biological value of the meat.

TABLE 1

Metabolic data showing the biological value of western beef when raw, canned, roasted and corned

RATION	MEAT Treatment	MEAT Cut	PERIOD	AVERAGE WT. OF RAT	N INTAKE	URINARY N	FECAL N	ENDOGENOUS N	METABOLIC N	DIGESTIBILITY	BIOLOGICAL VALUE
				gm	mg	mg	mg	mg	mg	%	%
Grass-fed	Raw	Round	A	90	87	35	11	12	9	95	72
			A	86	87	38	10	9	9	99	67
			A	85	87	31	14	10	8	93	74
			A	76	87	29	10	10	8	98	75
			A	82	87	33	10	10	9	98	73
										Average	73
			C	128	87	32	11	17	9	97	52
			C	124	87	28	10	16	9	99	56
			C	113	87	30	9	16	8	99	53
			C	110	87	26	9	18	8	99	91
			C	131	87	25	11	13	9	97	56
										Average	56
	Canned	Round	A	81	88	37	14	11	9	94	69
			C	120	88	32	13	15	9	94	79
Grass-fed	Raw	Rib	A	84	87	37	10	10	9	98	70
			C	128	87	28	10	16	8	98	56
	Roasted	Rib	A	88	86	35	9	11	8	99	71
			C	127	86	26	10	15	9	98	57
Grass to 60-day grain	Raw	Round	A	81	109	50	9	19	8	99	72
			C	112	123	44	9	28	8	100	55
	Corned	Round	A	77	109	55	11	20	8	97	66
			C	115	120	44	10	27	9	99	56
Grass to 90-day grain	Raw	Round	A	79	100	51	9	21	8	99	69
			C	112	113	47	10	29	8	99	85
	Corned	Round	A	78	103	55	9	20	7	98	66
			C	111	119	45	10	29	9	99	57
Grain-fed	Raw	Round	A	78	107	49	10	17	8	99	70
			C	114	121	43	10	25	9	99	55
	Corned	Round	A	75	107	52	8	16	8	99	66
			C	115	114	44	10	25	9	99	83

The biological values as calculated for each period differ considerably, but confirm the work of Mitchell ('28). Whether the value of period A or C or an average should be selected as the true biological value is open to question. This difference is interpreted by Chick et al. ('35) as a "nitrogen debt" incurred during specific protein starvation. Mitchell and Beadles ('37) suggested that the discrepancy was due to the fact that in the low nitrogen feeding period the endogenous level of nitrogen excretion had not been attained, thus increasing the biological value. More recently, Mitchell has recommended the use of a 7-day preliminary feeding period (Sahyun, '48). Differences in biological values between periods A and C may be due in part to incomplete adjustment of the animals to the experimental rations in the 5-day preliminary period used in this study.

Since this is a comparative study and the values for periods A and C vary rather widely, it would appear that the data obtained for the two periods should be treated and compared separately. The average biological values of raw beef round (73%, period A; 86%, period B) obtained in this investigation are somewhat comparable to that (76%) found by Mitchell and Beadles ('37). Morgan and Kern ('34), using the lower protein level of 7 instead of 10% and measuring the requirements for maintenance alone, obtained lower biological values for raw bottom round (67%) and found that cooking decreased these values further.

It will be noted that in period A canning lowered the average biological value of the beef protein from 73 to 69%. When the individual data were treated statistically according to the method of analysis of variance, the "F" value obtained, 3.44, was not as large as that required for significance, 5.12, in Snedecor's ('46) "F" table. However, the "F" value obtained is large enough to suggest that if more rats had been used in the study the lowered biological value of the canned meat obtained in this period might have been significant. When the data from period C were treated in the same manner it was found that the lowered value of the canned meat,

79% as compared to 86% for the raw, was significant at a 5% level with an "F" value of 9.15. Analysis of the data indicated that canning significantly lowered the digestibility of the protein from 97 and 98 to 94% in periods A and C respectively. The processes of roasting and corning did not bring about any significant change in either the biological value or digestibility.

When the data were analyzed further, no differences were found in the biological value and digestibility of the meat proteins from grain-fed and grass-fed animals. Similarly, in a previous study, the thiamine and riboflavin values of beef from grain-fed and grass-fed animals were comparable (Mayfield and Hedrick, in press).

SUMMARY

The effect of varying the type of feed and the subsequent changes produced by canning, roasting and corning on the digestibility and biological value of beef protein were studied.

The nitrogen balance technique with weanling rats was used for this investigation, following the method of Mitchell. When values for the periods were compared separately, statistical analysis of the data showed that in period C canning caused a slight but significant lowering in the biological value (86 to 79%). In period A, however, the difference (73 to 69%) was not significant. Digestibility values were significantly lowered by the canning process, from 98 to 94% in both periods. Roasting and corning did not change either the biological value or digestibility.

Beef round from grass-fed and grain-fed animals had similar biological and digestibility values.

ACKNOWLEDGMENT

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COMPARATIVE EFFECTIVENESS OF VITAMIN B_{12}, WHOLE LIVER SUBSTANCE AND EXTRACTS HIGH IN APA ACTIVITY, AS GROWTH PROMOTING MATERIALS FOR HYPERTHYRIOD ANIMALS[1]

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The relationship of the thyroid hormone to the nutrition of laboratory animals has been studied for some time (Drill, '43; Ershoff, '47a; Betheil, Wiebelhaus and Lardy, '47; Johnson, Hansen and Lardy, '48). It has been found both in this laboratory and by Ershoff ('47a, '47b) that rats ingesting desiccated thyroid gland require, in addition to all known vitamins, a growth promoting substance which is present in liver. The active principle of liver was found to be relatively heat stable and was not destroyed by autoclaving for one hour in solutions at pH 1, 7 or 10 (Betheil, Wiebelhaus and Lardy, '47).

In more recent work, the beneficial effects of anti-pernicious anemia (APA) extracts and of vitamin B_{12} have been noted. The similarity in properties between the growth promoting activity of liver and the APA factor indicated a possible re-

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lationship between the nutritional factor or factors required by hyperthyroid rats and the APA factor. Results are reported here which indicate that highly purified preparations of the APA factor are effective in *partially* preventing the growth retardation observed in rats fed thyroid substance. This finding may form the basis of an animal assay for a *growth promoting substance or substances* in APA concentrates. With the rat, a graded growth response is obtained with increasing amounts of the extracts equivalent to a daily intake of 0.01 to 0.1 USP units of the APA factor.

The growth promoting activity of various liver fractions and of other natural materials was also tested and is reported.

METHODS

The growth promoting activity of the materials studied was determined by the 4-week growth assay procedure using thyroid-fed rats previously described (Betheil, Wiebelhaus and Lardy, '47). Male, weanling, Sprague-Dawley strain rats weighing between 37 and 42 gm were grouped in lots (usually 6 rats) the average weights of which were equal. Negative (basal ration plus desiccated thyroid USP [2]) and positive (basal ration only) control groups were included in each experiment. Activity was determined by comparing growth on the thyroid-containing ration plus the supplement with that of the positive and negative controls. Using this growth assay the relative activity of the materials studied was usually compared with that obtained by the addition to the ration of a supplement of the 3 or 5% whole liver powder. This level of whole liver powder is insufficient to give a maximum response but it was used since it provides a sensitive estimate of the value of fractionation procedures on liver substance. It will be shown that liver powder at a 10% level completely reverses the growth inhibition caused by feeding desiccated thyroid at a level of 0.25% of the ration.

[2] Wilson.

The basal ration contained the following in parts by weight: casein[3] 22, sucrose 68.5, salts IV (Phillips and Hart, '35) 4.5, and corn oil 5. Vitamins were added to each kilogram of ration at the following levels: thiamine hydrochloride, 6 mg; riboflavin, 12 mg; nicotinic acid, 40 mg; pyridoxine hydrochloride, 6 mg; calcium pantothenate, 60 mg; choline chloride, 4 gm; *p*-aminobenzoic acid, 100 mg; biotin, 0.2 mg; folic acid, 0.5 mg; inositol, 2 gm; and 2-methyl-1, 4-naphthoquinone, 4 mg. Each animal received three drops of haliver oil USP[4] weekly. All rations were fed ad libitum.

EXPERIMENTAL RESULTS

Solubility of the growth promoting factor or factors of liver

Previous work (Betheil, Wiebelhaus and Lardy, '47) indicated the relative stability of the antithyrotoxic activity of liver. As a preliminary step in fractionation, an attempt was made to separate the active material from the bulk of the liver substance. Whole liver powder was repeatedly extracted with the following solvents: 60% aqueous ethanol, 95% ethanol, 1:1 ethanol-diethyl ether, chloroform. Extraction was continued with each respective solvent until the solvent layer contained no colored material. Chloroform extracts of whole liver substance were devoid of activity but each of the other solvent combinations tried removed some active material. In all cases the residue contained much of the active principle. Water extraction was likewise found to remove only *a part* of the activity. To determine whether the active material could be rendered soluble, water extraction was tried after autoclaving whole liver substance at pH 10 (Betheil, Wiebelhaus and Lardy, '47). The alkaline autoclaved whole liver powder was extracted with chloroform and the biologically inactive extract discarded. The residue was dried and powdered and then extracted repeatedly with distilled water at pH 6.8. The

[3] Vitamin test casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

[4] Abbott.

combined water extracts were cleared in a Sharples centrifuge to remove all suspended material and were concentrated to dryness under reduced pressure. The results of the feeding experiments are shown under experiment A in table 1. It is evident that only a part of the activity had been brought into solution and that the residue contained the bulk of the active material.

Variation in water solubility of the growth factor or factors is indicated by the relative activity of various batches of ex-

TABLE 1

Growth promoting activity of natural materials and solubility of the liver factor

SUPPLEMENT TO BASAL RATION + THYROID [1]	AVE. WT. AT 4 WEEKS		
	Exp. A	Exp. B	Exp. C
	gm	*gm*	*gm*
1. Positive control — no thyroid substance	211 ± 15.4 [2]	167 ± 23.2	183 ± 7.2
2. Negative control (basal ration + thyroid), no supplement	133 ± 13.5	122 ± 17.8	123 ± 6.8
3. 3% whole liver powder	191 ± 7.0		162 ± 1.0
4. Alkaline autoclaved W.L.P.[3]	168 ± 9.1		
5. Water extract of $CHCl_3$-extracted 4	148 ± 7.9		
6. Residue from 5	173 ± 10.3		
7. 2% Abbott liver extract powder 1:12	173 ± 11.5		
8. 1.5% liver powder 1:20 (Wilson)			145 ± 19.1
9. 1.0% liver powder 1:20 (Wilson)			141 ± 12.8
10. 0.5% liver powder 1:20 (Wilson)			133 ± 16.9
11. 1% skim milk powder		130 ± 10.3	
12. 10% skim milk powder		127 ± 11.8	
13. 1% dehydrated canned tomatoes		131 ± 14.8	
14. 10% dehydrated canned tomatoes		149 ± 12.6	

[1] Desiccated thyroid fed at a level of 0.38% in experiment C, at 0.25% in experiments A and B. Six rats per group.

[2] Standard deviation.

[3] W.L.P. = Whole liver powder. All liver fractions were fed at concentrations equivalent to 3% whole liver powder.

tracts from fresh liver.[5] One lot was found to be as active as whole liver substance (Betheil, Wiebelhaus and Lardy, '47). Since then a second lot has been found devoid of activity. Ershoff ('47b) has stated that liver powder 1:20 had little or no antithyrotoxic activity. Results obtained in this laboratory with a third lot of liver powder 1:20 are described under experiment C, table 1. An extract from another source [6] was found only partially effective (group 7, table 1). It seems possible that variations in time between slaughter and extraction, with corresponding variations in autolysis, may be responsible for these wide differences in activity.

Growth promoting activity of anti-pernicious anemia preparations for hyperthyroid animals

The growth promoting material in liver resembles the APA factor in thermo-stability, in resistance to acid, and, to some extent, in its partial solubility in aqueous alcoholic solutions. In earlier work Sure and Buchanan ('37) showed that a liver extract used in the treatment of pernicious anemia had a beneficial effect on hyperthyroid animals, and attributed this effect to its content of B vitamins. Recently Jaffe and Elvehjem ('47) and Nichol et al. ('47) have reported a growth stimulating effect of APA preparation when fed to animals receiving a corn-soybean ration.

It has been found that purified liver extracts rich in APA activity exhibited growth promoting activity when injected into rats receiving desiccated thyroid substance in their ration.

In a preliminary experiment, the intraperitoneal injection of one USP unit of APA liver extract [7] per day into rats which had been fed a ration containing 0.38% desiccated thyroid USP for 5 weeks resulted in growth stimuation. The growth curves of these animals had previously reached a plateau and a loss in weight had begun. The liver extract immediately arrested

[5] Liver powder 1:20, Wilson.

[6] See footnote 4, page 497.

[7] Reticulogen No. 360, Lilly, 20 USP units per milliliter.

TABLE 2

Growth promoting activity of anti-pernicious anemia liver extracts

SUPPLEMENTS TO BASAL RATION + THYROID [1]	AVERAGE WEIGHT AT 4 WEEKS: Exp. A	Exp. B		Exp. C		Exp. D
	gm	gm		gm		gm
1. Positive control — no thyroid substance	171 ± 16.5 [2]	211 ± 15.4		171 ± 8.7		183 ± 20.8
2. Negative control (basal ration + thyroid), no supplement	124 ± 17.6	133 ± 13.5		130 ± 6.7		147 ± 4.5
3. 3% whole liver powder	158 ± 9.5	191 ± 7.0		159 ± 7.8		168 ± 1.2
4. 10% whole liver powder						188 ± 17.4
5. 300 μg vitamin B_1 intra-peritoneally/day		133 ± 10.1				
		Lilly product	Sharp and Dohme product	Lilly product	Sharp and Dohme product	
6. APA concentrate [3] 0.005 units/day				131 ± 14.3	130 ± 13.1	
7. APA concentrate 0.01 units/day				136 ± 10.5	133 ± 14.9	
8. APA concentrate 0.025 units/day				144 ± 3.3	136 ± 9.0	
9. APA concentrate 0.05 units/day		150 ± 14.1	152 ± 8.3	158 ± 8.7	139 ± 7.8	
10. APA concentrate 0.1 units/day		150 ± 8.0	164 ± 13.0	143 ± 7.2	152 ± 9.1	161 ± 15.3
11. APA concentrate 0.5 units/day		168 ± 16.9	158 ± 4.0			
12. APA concentrate 1.0 units/day	149 ± 13.4	157 ± 9.0	161 ± 8.4			
13. APA concentrate 2.0 units/day		162 ± 10.6	160 ± 4.3			
14. APA concentrate 1.0 units/day (4th week only)	137 ± 16.5					
15. Formylfolic acid, 100 μg/day.						147 ± 19.2

[1] Desiccated thyroid fed at 0.38% of the ration in experiment A, at 0.25% in experiments B, C and D. Six rats per group.

[2] Standard deviation.

[3] All APA concentrates were administered by intraperitoneal injection.

the weight loss and growth was resumed. A control group receiving only the thyroid containing ration continued to lose weight during the two-week experimental period.

The results of more extensive studies are shown in table 2. Preparations containing APA activity were almost as effective in overcoming growth retardation as was 3% whole liver substance. APA preparations were very effective when given concurrently with desiccated thyroid, and also elicited a growth response when given after thyroid had been fed for three weeks (experiment A, table 2).

In another experiment the effect of Reticulogen on recovery from thyrotoxicosis was studied. Male rats were kept on a thyroid containing ration (0.25% desiccated thyroid) for 7 weeks. Very few of the animals survived. Those which did were divided into two groups, equal with respect to average weight, of 6 animals each. All animals were losing weight at that time. The two groups were placed on the basal ration and one group was supplemented with one unit of Reticulogen per day intraperitoneally. All animals grew after removal of desiccated thyroid from the ration. However, Reticulogen-supplemented animals grew better during the first two weeks of the recovery period, averaging a 70 gm gain, while the unsupplemented group gained only 57 gm during this period. After three weeks the rats in all groups averaged the same in weight. Apparently this animal can rapidly readjust itself once the stress induced by thyroid administration is removed. After three weeks on a thyroid-free ration, the APA preparation had no effect on growth.

To determine whether the growth promoting activity of APA preparations paralleled the anti-pernicious anemia potency of the material, graded levels of two different preparations [8] were administered intraperitoneally to animals receiving the thyroid-containing ration. A group receiving 300 µg of thiamine was included in the experiment since Retic-

[8] Lilly Reticulogen and Sharp and Dohme Refined Liver Extract (No. 2505, 15 USP units/ml) were used.

TABLE 2

Growth promoting activity of anti-pernicious anemia liver extracts

THYROID	AVERAGE WEIGHT AT 4 WEEKS			
	Exp. A	Exp. B	Exp. C	Exp. D
	gm	*gm*	*gm*	*gm*
		211 ± 15.4	171 ± 8.7	183 ± 20.8
		± 13.5	130 ± 6.7	147 ± 4.5
			159 ± 7.8	168 ± 1.2
				188 ± 17.4

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were divided into
distribution of sexes.
purified liver extract[9]
USP units per day. The
of Reticulogen

ulogen contains added thiamine. All dilutions of liver extracts were made with physiological saline.

The results are shown under experiments B and C, table 2. Supplementation with thiamine had no effect. The maximum effect was obtained with levels of liver extract between 0.05 and 0.5 USP unit. At all higher levels both preparations gave approximately the same growth stimulation over that of the negative control.

Experiment C, table 2, shows the effect on growth of lower levels of the APA preparations. The extracts used in this experiment were from different batches than those used in 2A. From the data it appears that a maximum response is obtained with approximately 0.1 unit of liver extract.

Effect of liver extracts on thyrotoxic rabbits

To determine whether the antithyrotoxic effect of APA preparations could be observed in animals other than the rat, an experiment was conducted with rabbits injected with thyroxine. Weanling rabbits from a commercial breeder were fed the following basal ration (parts by weight) for a period of 6 weeks before beginning treatment: ground oats, 11; ground yellow corn, 36; soybean meal, 46; corn oil, 5; cystine, 0.3; $CaHPO_4$ 0.92, $CaCO_3$ 0.6, NaCl (iodized) 0.44, $MnSO_4 \cdot 4H_2O$ 0.04. Vitamins were added at the levels used in the purified ration described above. The ration was pelleted before use.

Thyroxine was administered intraperitoneally at the following rate: first week, one injection of 1 mg/kg; second week, 1 mg/kg on alternate days; thereafter injections of 0.5 mg/kg were made every third day for 4 additional weeks. At the beginning of the third week the rabbits were divided into two groups of equal size and with equal distribution of sexes. One group of 7 animals received a purified liver extract[9] intraperitoneally at a level of two USP units per day. The experiment was terminated after 4 weeks of Reticulogen

[9] Reticulogen, Lilly.

supplementation. The liver extract strikingly improved the survival of these thyrotoxic rabbits. Only one animal in the control group survived the 6-week period of thyroxine administration. In the group given liver extract, one rabbit died on the second day of therapy while the other 6 lived through the experimental period. Thyroxine administration caused a growth retardation in all animals and liver extract was not appreciably effective in reversing the retardation. Positive control animals not receiving thyroxine continued to grow well and all survived. Subsequent to our original finding of the beneficial effect of APA extracts on thyrotoxic rats. Robblee, Nichol, Cravens, Elvehjem and Halpin ('48) obtained similar results with chicks fed thyroid active materials.

Experiments with folic acid

The work of Jacobson and Good ('47) suggested that folic acid may be a precursor of the APA factor. An attempt was therefore made to determine whether the "intrinsic" factor of the normal stomach could convert folic acid into an antithyrotoxic mateiial. Folic acid (1 mg/ml) was incubated with a hog stomach preparation [10] and, in a second experiment, with fresh human gastric juice (pH 1.7). The enzyme solutions were adjusted to pH 7.0 with NaOH and incubated for 48 hours at 37°C. Microbiological assays [11] with *Streptococcus faecalis* (American Type Culture Collection No. 8043) demonstrated that no significant destruction of folic acid had occurred. The enzyme-treated folic acid was assayed (100 μg/day) with thyroid-fed rats as described above. Neither the incubated mixture nor the original enzyme materials had any beneficial effect on the growth of thyrotoxic rats. Formylfolic acid prepared by the method of Gordon et al. ('48) was likewise found to have no beneficial effect on rats fed a thyroid-containing ration (experiment D, table 2).

[10] Ventriculin, Parke Davis.

[11] Kindly done by Dr. O. E. Olson and Mr. E. Fager.

Other sources of the antithyrotoxic factor or factors

Previous work (Betheil, Wiebelhaus and Lardy, '47) had indicated the presence of some antithyrotoxic substance in yeast in addition to the known vitamins. Results of assays of other natural materials are included in table 1. Skim milk powder was a poor source of the factor or factors, while canned tomatoes exhibited some growth promoting activity. In another experiment a preparation of fish solubles was found to be almost as active in promoting the growth of thyrotoxic rats as was dried whole liver substance. Nichol et al. ('47) have reported a growth stimulation due to fish soluble components with chicks fed a supplemented natural ration. In our studies with the rat no source of the factor has proved more potent than dried whole liver substance. In two experiments 10% bovine blood fibrin was inactive.

The effect of vitamin B_{12} on the hyperthyroid rat

With the isolation of crystalline vitamin B_{12} from liver (Rickes et al., '48) and the demonstration of its potent activity in eliciting hemopoietic responses in pernicious anemia patients (West, '48), it was of interest to determine whether this substance possessed growth promoting activity for the hyperthyroid rat. Ott and co-workers ('48) have recently reported the growth stimulatory activity of vitamin B_{12} for chicks.

Two types of preparations [12] were available. One was the pure crystalline material, the other a concentrate containing 2 mg vitamin B_{12} per pound in a mixture of soybean flour and charcoal. Crystalline vitamin B_{12} was administered by intraperitoneal injection, while the concentrate was added to the thyroid-containing ration. Supplements were administered for 42 days. The results are shown in table 3. It can be seen that vitamin B_{12} had pronounced growth promoting properties

[12] We are indebted to Dr. D. F. Green of Merck and Company, Inc., for generously making these preparations available.

at the levels used (0.2 µg intraperitoneally daily and 30 µg per kilogram of ration). In an earlier experiment, lower levels were found to be much less effective. Since an optimum amount of liver extract gave no better growth than vitamin B_{12}, it seems that all or most of the growth promoting activity of APA liver extracts resides in their vitamin B_{12} content. Conversely, it appears therefore that the vitamin B_{12} requirement of the hyperthyroid rat lies between approximately 0.1 and 0.2 µg per day when the material is administered parenterally.

TABLE 3

Effect of vitamin B_{12} on the growth of hyperthyroid rats

LOT NO.[1]	REGIMEN	AVERAGE WEIGHT			
		26 days		42 days	
		gm		*gm*	
1	Basal ration	191 ± 11.1[2]	(8)[3]	281 ± 19.9	(8)
2	Basal + 0.25% desiccated thyroid USP	149 ± 18.5	(6)	183 ± 21.1	(4)
3	2 + 0.2 µg vitamin B_{12} injected intraperitoneally daily	173 ± 12.1	(8)	222 ± 19.9	(7)
4	2 + 0.10 unit Reticulogen (Lilly) injected intraperitoneally daily	166 ± 9.9	(8)	213 ± 11.3	(6)
5	2 + 5% whole liver powder in the ration	180 ± 12.4	(8)	247 ± 13.7	(6)
6	2 + 30 µg vitamin B_{12} concentrate per kg of ration	163 ± 14.5	(8)	229 ± 22.2	(6)

[1] Eight rats per lot.
[2] Standard deviation.
[3] Number of animals surviving at that time.

Iodinated casein and thyrotoxicosis

The possibility was investigated of producing the hyperthyroid syndrome in rats with a substance more readily available than desiccated thyroid. Iodinated casein [13] was fed at

[13] Protamone, Cerophyl Laboratories, Inc., Kansas City, Missouri.

various level to male rats fed the purified diet, with the results shown in table 4; a graded retardation of growth was obtained with increasing levels. Although it is much less effective than thyroid substance when fed at levels equal with respect to iodine supply, iodinated casein appears to be a satisfactory agent for the production of experimental hyperthyroidism.

TABLE 4

Growth retardation in rats: iodinated casein compared with desiccated thyroid

SUPPLEMENT TO BASAL RATION	AVERAGE WT. AT 4 WEEKS[1]
	gm
None	189 ± 11.0[2]
0.38% desiccated thyroid	119 ± 5.6
0.03% iodinated casein	163 ± 12.7
0.06% iodinated casein	150 ± 10.8
0.10% iodinated casein	149 ± 7.8
0.20% iodinated casein	133 ± 4.2
0.30% iodinated casein	112 ± 9.9

[1] Three rats per lot.
[2] Standard deviation.

DISCUSSION

The results obtained in the present study demonstrate that in thyrotoxicosis rats require one or more growth factors which are not needed by normal rats fed highly purified diets. Purified liver extracts rich in the APA factor are a good source of this growth factor. As little as 0.0005 ml (about 50 µg dry matter) daily produced an appreciable response (table 2). The thyrotoxicosis produced by the thyroid-containing ration is not accompanied by an anemia. Hemoglobin levels of animals showing the typical growth depression are normal. It may well be that the growth response reported here is due to some constituent in the liver extract other than the APA factor. However, it has been found that liver fractions containing less APA activity on a dry weight basis were also less active in supporting the growth of hyperthyroid rats. If this growth stimulating factor is the same as that required for

blood cell maturation in human beings, these observations may be the basis for an animal assay for the APA factor. The graded growth response obtained with increasing doses of purified liver extracts (table 2) indicates the possibility of such an assay.

From evidence available at present it cannot be definitely stated whether or not vitamin B_{12} and the anti-pernicious anemia factor are identical. Further clinical testing will be required to determine whether the crystalline material will cure the neurological as well as hematological lesions which are typical of Addisonian pernicious anemia. Vitamin B_{12} is effective in ameliorating the growth retardation which results from the feeding of desiccated thyroid (table 3). The growth promoting activity of vitamin B_{12} in adequate amounts is of the same order of magnitude as that of APA extracts, and a close relationship between the two materials in the nutrition of the rat is indicated. The above data (table 3) definitely show a requirement for vitamin B_{12} in the nutrition of the hyperthyroid rat. The normal rat probably also requires the vitamin but the hyperthyroid animal allows a clearer demonstration of this need.

While a number of natural materials — tomatoes, fish soluble components and yeast — have been found to promote the growth of thyroid-fed rats, *none of these is as effective as liver*. In view of the fact that the pernicious anemia patient has difficulty in consuming sufficient liver to supply his needs for the APA factor, it is not surprising that these other *possible* sources of the factor have been overlooked.

There is some evidence of the dual nature of the growth factor present in whole liver powder. The solvent fractionation experiments presently reported indicate that in spite of exhaustive extraction of the liver powder only a part of the activity can be brought into solution. The possible occurrence of a bound form of the growth factor is indicated.

In some experiments (table 2) whole liver gave a greater growth response than purified extracts. Regardless of the number of factors required by the rat fed thyroxine-containing

rations, the technique just described should be well suited for studying additional growth promoting substances. The basis for this view has been previously discussed in detail (Betheil, Wiebelhaus and Lardy, '47).

SUMMARY

1. Rats fed thyroid-containing rations require for growth a factor or factors present in liver, fish soluble components and tomatoes. Skim milk and fibrin contain little or none of this factor.

2. Highly purified liver extracts rich in the anti-pernicious anemia principle carry the factor.

3. APA preparations had a significant effect on longevity when injected into hyperthyroid rabbits.

4. The significance of these observations in the development of an assay for the APA factor is noted.

5. Vitamin B_{12} was found to be a growth factor for the hyperthyroid rat.

6. Treatment of folic acid with good sources of the "intrinsic factor" did not result in the formation of a growth promoting substance. "Formylfolic acid" was likewise inactive in our assay.

7. Iodinated casein may be used to produce the thyrotoxic syndrome.

8. Exhaustive extraction of whole liver powder with 60% ethanol, 95% ethanol, 1:1 ethanol: ether or water brought only part of the active material into solution. These observations as well as others indicate the possible dual nature of the material which, in addition to the known vitamins, supports the growth of animals fed thyroxine-containing rations.

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THE SULFUR AMINO ACID REQUIREMENT OF THE INFANT[1]

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ONE FIGURE

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In our studies on the amino acid requirements of the adult (Albanese and associates, '43), we found that subjects on a methionine-deficient diet developed negative N balances which were promptly abolished by the addition of a methionine supplement, and concluded therefore that methionine is a human dietary essential. The role of cystine, however, remained in doubt, for parallel experiments with a cystine-deficient diet gave equivocal results in regard to nitrogen balance. This doubt was resolved by a subsequent investigation (Albanese and co-workers, '44) in which it was found that two healthy male subjects who developed negative nitrogen balances on a diet deficient in both sulfur amino acids (methionine and cystine) were restored to nitrogen equilibrium with a supplement to the doubly deficient diet of 18.8 mg of DL-methionine per kilogram of body weight. How far this intake is above the minimum methionine requirement could not be stated because various levels of supply below this amount were not tested.

With this information as a rough base, however, and the known fact that on a body weight basis the nitrogen require-

[1] The work described in this report was supported by grants from the National Livestock and Meat Board, Mead Johnson and Company, and National Dairy Products, Inc.

ments of the infant are about 5 times greater than those of the adult, it might be deduced that 5 times this adult value, or 94 mg of methionine per kilogram should provide the infant with more than an adequate supply of the sulfur amino acids. Nevertheless, the desirability of determining this requirement by direct measurements (which are reported here) rather than by inference is pointed up (a) by the claim that sulfur amino acids may constitute a limiting dietary factor in cow's milk formulas and (b) by our assay of isoleucine requirements (Albanese et al., '48) which indicated that infant needs for the individual amino acids do not always follow the amino acid composition of the milk proteins.

METHODS AND DIETS

In this as in previous investigations (Albanese et al., '48), the requirement of an essential amino acid was estimated on the basis of the amount needed to restore to normal physiological levels the nitrogen retention and rate of body weight gain of subjects previously maintained on a deficient diet. Previous experiences indicated the desirability of the following regimen: For the week preceding the deficient period the subjects were maintained on a "complete" diet, i.e., the deficient diet supplemented with cystine and methionine. The deficient diet was fed for the following three weeks to all 5 subjects. Subsequently the diet of three subjects was supplemented with L-methionine in graded amounts to a maximum of 3% of the protein intake. At the same time the diet of the other two subjects was supplemented with 1% L-cystine alone and then with 1% L-cystine and increasing amounts of L-methionine. Seven-day intervals elapsed between all dietary changes tested.

The observations reported here were made on normal healthy male infants who were given the purified diets in 5 feedings daily at the rate of approximately 100 cal. per kilogram of body weight and 500 mg of ascorbic acid together with 15 drops of oleum percomorphum daily. As noted previously, the diet periods were of 7 days' duration and were consecutive, but collections of excreta were omitted on week-ends. The sub-

TABLE 1

Composition of diets

(All diets fed at the rate of 100 cal. and 3.5 gm of protein per kilogram per day)

COMPONENTS	DIET A	DIET B	DIET C	DIET D	DIET E	DIET F	DIET G	DIET H
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Oxidized casein hydrolysate[1]	3.30	3.41	3.42	3.40	3.38	3.40	3.38	3.36
L-Tryptophane	0.06	0.06	0.06	0.06	0.06	0.06	0.06	0.06
L-Cystine	0.04	0.00	0.00	0.00	0.00	0.04	0.04	0.04
L-Methionine	0.10	0.00	0.02	0.04	0.06	0.00	0.02	0.04
Brewers' yeast[2]	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Olive oil	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Dextri-maltose no. 2	9.60	9.60	9.60	9.60	9.60	9.60	9.60	9.60
Arrowroot starch	2.30	2.30	2.30	2.30	2.30	2.30	2.30	2.30
Salt mixture[3]	1.60	1.60	1.60	1.60	1.60	1.60	1.60	1.60
Water	78.00	78.00	78.00	78.00	78.00	78.00	78.00	78.00
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Estimated L-cystine content (mg)	50	10	10	10	10	50	50	50
Estimated L-methionine content (mg)	125	25	45	65	85	25	45	65

[1] N × 6.25 = *grams of protein containing 0.10% of cystine and 0.28% of methionine by chemical analysis.*

[2] Kindly supplied by Mead Johnson and Company *and found by chemical analysis to contain 1.3% of cystine and 2.0% of methionine.*

[3] The salt mixture employed had the following composition (measured in grams): $FeSO_4$ 0.9, NaCl 6, Ca gluconate 48, $Ca(OH)_2$ 12, KH_2PO_4 7, KCl 6, and MgO 0.1.

jects were partially immobilized by specially designed abdominal supports, which also held the urinary adapters in place. Twenty-four hour urine specimens were collected in bottles containing 10 ml of 15% (by volume) HCl and 1 ml of 10% alcoholic thymol. The feces were collected in 19 cm porcelain evaporating dishes which were kept in place by a properly shaped excavation in the mattress, and the daily stools for each period were accumulated under refrigeration in wide-mouth jars containing 200 ml of 70% alcohol. The subjects were weighed daily during the course of the experiment.

The composition of the diets used in this study is shown in table 1. The diets provided approximately 100 cal. per 100 gm, the percentage caloric distribution being as follows: Protein, 14; fat, 36; carbohydrate, 50. The protein moiety of the sulfur amino acid-deficient diet was prepared by tryptophan reinforcement of a hydrogen peroxide oxidized hydrolysate of casein (as described by Albanese et al., '44) which was found by chemical analysis to contain 0.28% of methionine and 0.10% of cystine. The protein equivalent of the final product was estimated as N × 6.25 and it was supplemented with 1.5% of L-tryptophan to replace that which was destroyed during acid hydrolysis. The cystine and methionine supplements were added to the diet as shown in table 1. Owing to uncertainties regarding the complete human requirements of B complex vitamins, brewers' yeast was employed instead of a mixture of the synthetically available vitamins. The quantities of cystine and methionine derived from this source were found by chemical analyses to be 6.5 and 10.0 mg, respectively, per gram. Thus, the amounts of the sulfur amino acids per kilogram of infant body weight provided by the diets can be estimated roughly (table 1). The final nitrogen content of each batch of diet was determined by micro-Kjeldahl analysis.

The data on nitrogen retention were calculated from the results of nitrogen determinations of the 24-hour urine collections, analyses of the pooled feces from each period, and computations of the daily N intake based on food consumption records and the known nitrogen content of the diets.

TABLE 2

Effect of dietary methionine on the nitrogen retention, body weight and blood proteins of the infant

INITIAL AGE AND WEIGHT OF SUBJECT	CATEGORY OF INTEREST	DIET A	DIET B	DIET B	DIET B	DIET C	DIET D	DIET E	DIET A
	Cystine intake (mg/kg/day)	50	15	15	15	15	15	15	50
	Methionine intake (mg/kg/day)	125	25	25	25	15	65	85	125
D.C.,	N intake (gm/day)	3.30	3.26	3.14	3.10	3.14	3.14	3.14	3.24
male,	N retention (mg/kg/day)	106	93	53	28	68	55	90	120
11 mos.	Weight change (gm/day)	+24	+14	−10	−7	0	+7	+20	+19
7.193 kg	Total plasma proteins (gm%)	6.45	6.35	6.25	6.19	5.76	5.12	5.38	5.40
	Hemoglobin (gm%)	10.10	9.90	9.90	10.80	10.20	10.60	11.10	11.10
A.H.,	N intake (gm/day)	4.55	4.28	4.18	4.08	4.18	4.18	4.18	4.14
male,	N retention (mg/kg/day)	116	82	77	47	72	19	98	110
11 mos.	Weight change (gm/day)	+20	−35	+4	+4	0	0	+17	+22
7.346 kg	Total plasma proteins (gm%)	6.11	6.68	6.92	5.60	6.13	5.73	5.82	6.80
	Hemoglobin (gm%)	11.00	10.60	10.50	11.10	10.60	10.80	10.50	11.60
R.R.,	N intake (gm/day)	1.78	1.76	1.78	1.79	1.75	1.80	1.78	1.78
male,	N retention (mg/kg/day)	96	14	17	−41	30	−6	99	112
4 mos.	Weight change (gm/day)	+24	−14	−35	−24	0	−13	+14	+27
3.538 kg	Total plasma proteins (gm%)	5.85	6.12	5.66	6.06	5.32	5.82	5.72	6.13
	Hemoglobin (gm%)	10.40	9.60	8.90	9.80	9.80	9.40	10.30	10.80

Blood samples were collected over lithium oxalate by vena puncture on the last day of each diet period. The hemoglobin concentration of these specimens was determined colorimetrically in the Klett-Summerson photoelectric colorimeter. The total plasma proteins, albumin, globulin and non-protein N were determined by the procedure described by some of the present authors (Albanese, Irby and Saur, '46).

RESULTS AND DISCUSSION

The results obtained in the first experiment (table 2) show that the feeding of the doubly deficient diet B caused a prompt and sustained fall of the daily weight gain and nitrogen retention below the control levels of all three subjects. In a growing organism such as the infant, a drop from the nitrogen retention values characteristic of the individual must be given the same interpretation as the establishment of a negative N balance in the adult, namely, that diet B is lacking in essential dietary components. This inference is supported by the concomitant decrements in rate of weight gain. It will be seen in table 2 that following the deficient period all three subjects were given in turn diets C, D, E and A, which provided the infants with 45, 65, 85 and 125 mg of L-methionine per kilogram of body weight, respectively. Examination of the resulting data discloses that the nitrogen retention and weight coefficients of the subjects were restored to normal levels by diet E but not by diets C and D, and the values of these criteria attained with diet E were not augmented by the refeeding of control diet A. From these observations it may be deduced that the sulfur amino acid needs of the infant can be adequately met by an intake of 85 mg of L-methionine and 15 mg of L-cystine per kilogram per day.

In order to assess the dispensability of cystine and its sparing action on the methionine requirements, a second experiment designed to elucidate both of these points was performed. In this experiment two subjects (table 3) were fed the cystine-supplemented diet F for one week following the three weeks of the doubly deficient regimen. And, since the cystine supple-

TABLE 3

Effect of dietary cystine and methionine on nitrogen retention and body weight and blood proteins of the infant

INITIAL AGE AND WEIGHT OF SUBJECT	CATEGORY OF INTEREST	DIET A	DIET B	DIET B	DIET B	DIET F	DIET G	DIET H	DIET A
	Cystine intake (mg/kg/day)	50	15	15	15	50	50	50	50
	Methionine intake (mg/kg/day)	125	25	25	25	25	45	65	125
R.M.,	N intake (gm/day)	2.90	2.80	2.90	2.90	2.90	2.90	2.90	2.90
male,	N retention (mg/kg/day)	102	33	35	13	37	82	109	109
6 mos.	Weight change (gm/day)	+21	−21	−14	−14	21	−13	+18	+20
5.167 kg	Total plasma proteins (gm%)	5.70	6.38	6.23	6.43	6.26	4.82	5.42	5.83
	Hemoglobin (gm%)	11.20	8.20	8.70	9.40	10.10	10.20	9.90	10.90
H.G.,	N intake (gm/day)	3.63	3.83	3.52	3.54	3.54	3.54	3.54	3.54
male,	N retention (mg/kg/day)	111	92	47	16	41	99	128	120
8 mos.	Weight change (gm/day)	+14	−31	−21	0	−7	+14	+13	+14
7.560 kg	Total plasma proteins (gm%)	6.55	6.71	6.67	6.70	6.72	6.67	6.85	6.83
	Hemoglobin (gm%)	9.60	10.10	9.90	10.60	8.50	10.40	9.90	11.10

ment (35 mg per kilogram) failed to raise the nitrogen retention and weight gain induced by deficient diet B, it is concluded that cystine is not a dietary essential for the infant. In the subsequent week the cystine-supplemented diet F reinforced with 20 mg of L-methionine per kilogram (diet G) was fed and some improvement in the growth function was noted. However, the complete return to normal physiological N retention and weight gain values was achieved only in the following week when diet H was fed, which provided the infants with a total of 65 mg of methionine and 50 mg of cystine per kilogram of body weight. That these amounts of the sulfur amino acids were optimum is suggested by the observation that the growth criteria were not augmented by the feeding of control diet A in the last week of the experiment.

Since diet H in this experiment and diet E of the previous experiment appear to be equally capable of restoring N retention and weight gains to normal levels by subtraction of the amounts of cystine and methionine contained, respectively, in the diets, it can be deduced that in the presence of adequate quantities of methionine, 35 mg of cystine can spare approximately 20 mg of methionine. In terms of amino acid sulfur, 9 mg of cystine S are equivalent to about 4 mg of methionine S. This computation indicates that about 22% of the methionine requirement of the infant can be met by cystine, a figure which compares favorably with the 16% found by Womak and Rose ('41) for the immature rat.

In considering some of the implications of this study, it occurred to us that the results presented here might be of value in clarifying the clinical claim that the nutritive virtues of human milk over cow's milk are due to the higher sulfur amino acid content of the former. On the basis of calculations from the available data on the sulfur amino acid content of breast milk and various estimates of the intake of breast milk by infants of different ages (fig. 1), it would appear that even on the most liberal intake estimates breast milk provides a very meager margin of safety during the early months of life, and that it does not provide an adequate quantity of the sulfur

amino acids in the latter part of the first year. On the same basis cow's milk formulas, on the other hand, seem to provide adequate amounts of sulfur amino acids. These calculations support the view (albeit tentative since the calculations are based on relatively short-term experiments) that the claimed superior biological value of breast milk cannot be due in any great measure to the sulfur amino acid content of this food.

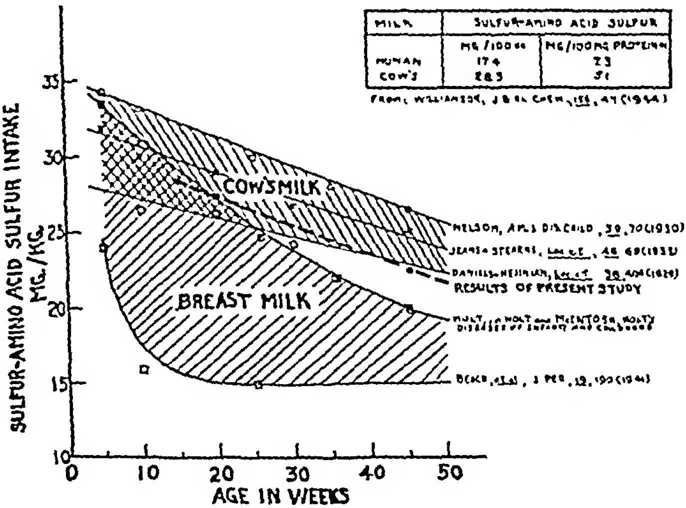

Fig. 1 Schematic comparison of the sulfur-containing amino acid sulfur provided by cow's milk formulas and by human milk with reference to the measured requirements of the infant.

Some support for this view can also be derived from the data reported by Gordon and his associates ('47), which point to the conclusion that mixtures of cow's milk will produce larger gains in weight in premature infants than will human milk.

SUMMARY

The sulfur amino acid requirements of 5 normal male infants (4–11 months of age) were studied. In three infants who received no cystine supplements the nitrogen retention and body weight gains were restored to normal levels by an intake of 85 mg of L-methionine per kilogram of body weight. In two

infants who received a 1% L-cystine supplement the nitrogen retention and weight change were restored to normal values by an intake of 65 mg of L-methionine per kilogram of body weight. The implications of these findings are discussed in reference to the nutritional virtues of human and cow's milk.

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TRYPTOPHAN AND NICOTINAMIDE IN THE NUTRITION OF THE ANIMAL MICROORGANISM, TETRAHYMENA[1]

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The many reports which have appeared in the literature during the past few years leave little doubt that the higher animals (rat, pig, dog, human) develop niacin deficiencies on diets low in tryptophan, and that the deficiency can be overcome by the addition of either niacin or tryptophan to the diet. Investigators have not been in agreement, however, regarding the site of niacin synthesis. It has been pointed out a number of times that the intestinal microflora may be responsible for the conversion of tryptophan to niacin rather than the animal *per se*. Recently Mitchell, Nye and Owen ('48) produced strong evidence that the rat is capable of niacin synthesis when they found that the parenteral administration of hydroxyanthranilic acid, an intermediate in the chain of reactions from tryptophan to niacin in mutant Neurospora (Mitchell and Nye, '48; Bonner, '48), resulted in a greatly increased N^1-methylnicotinamide excretion. On the other hand, Ellinger and Kader ('47) have demonstrated the importance of the microflora in niacin synthesis.

It seemed important to us to examine the problem of niacin synthesis in a given animal under conditions where the results could be unequivocally assigned to the responsibile mecha-

[1] Aided by a grant from the William-Waterman Fund for the Combat of Dietary Diseases.

nisms, within the animal, ruling out all possibilities of contaminating organisms. It has been possible to examine at the same time the effects of corn extract for a possible "pellagragenic agent." The results of these experiments, together with the short report by Schultz and Rudkin ('48) on "germ-free" Drosophila, make it apparent that not all animals possess the ability to synthesize niacin, and that the amount of tryptophan included in the diet in no way alters the situation.

TABLE 1

Standard supplements to the amino acid media
(All amounts are given in $\mu g/ml$ of final medium.)

Dextrose	1000	Ca pantothenate	0.10
Sodium acetate	1000	Pyridoxine HCl	1.00
Tween 85 [1]	700	Pyridoxal HCl	0.10
$MgSO_4 \cdot 7H_2O$	100	Pyridoxamine HCl	0.10
K_2HPO_4	100	Riboflavin	0.10
$CaCl_2 \cdot 2H_2O$	50	Pteroylglutamic acid	0.01
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	25	Biotin (free acid)	0.0005
$FeCl_3 \cdot 6H_2O$	1.25	Thiamine HCl	1.0
$CuCl_2 \cdot 2H_2O$	5	Choline chloride	1.0
$MnCl_2 \cdot 4H_2O$	0.50	Protogen [2]	0.375
$ZnCl_2$	0.05	Guanylic acid	30
		Adenylic acid	20
		Uracil	10
		Cytidylic acid	25

[1] Furnished by the Atlas Powder Co., Wilmington, Delaware.
[2] Supplied through the courtesy of Dr. E. L. R. Stokstad. For reference to this growth factor see Stokstad, Hoffman, Regan, Fordham and Jukes ('49) and Kidder and Dewey ('49a).

METHODS

The animal used in these studies was the ciliated protozoan, *Tetrahymena geleii*, strain W. This organism has been grown in pure (bacteria-free) culture in this laboratory for many years and its nutritional requirements are well known (Kidder and Dewey, '49a). In these investigations amino acids were used as the source of nitrogen, but these were varied to simulate some of the test diets used in similar studies on rats. This was done in order to obtain results which could be compared

with the same type of work on the higher animals. Thus 9%, 12%, 18% and 24% casein levels were simulated, the individual amino acids being added according to the published data on composition of casein (Hawk, Oser and Summerson, '47). All diets contained the various ingredients listed in table 1. Nicotinamide was used in varying concentrations, as will be indicated.

Cultures were incubated in a slanted position at 25°C. for 72 hours in 125 × 15 mm Pyrex test tubes and growth was measured turbidimetrically (Kidder and Dewey, '48a). Only the results of third serial transplants were considered. Experimental series were set up in triplicate and the individual tubes averaged for the final result. Each of the experiments was repeated varying numbers of times.

EXPERIMENTAL

Tetrahymena requires both tryptophan (Kidder and Dewey, '45, '48b) and niacin (Kidder and Dewey, '49b). If there is ability on the part of the organism to synthesize any niacin from tryptophan (or the reverse) then it should be possible to demonstrate sparing action, if not replacement. Accordingly, dose response experiments were conducted to determine the amount of nicotinamide required for half-maximum growth in the presence of increasing amounts of tryptophan. The amino acid levels for this set of experiments were the same as in simulated 12% casein (with tryptophan omitted). It was found that as the tryptophan was increased from a suboptimum amount (2 μg/ml) to a supraoptimum amount (28 μg/ml) there was a slight, but doubtfully significant, increase in the niacin requirement. There was certainly no sparing of niacin by high levels of tryptophan, which indicates clearly that Tetrahymena cannot synthesize the vitamin under these conditions.

In the light of reports regarding the effects of amino acids other than tryptophan on the apparent synthesis of niacin by the rat or the rat microflora (Ellinger and Kader, '47), an extensive series of experiments was conducted in which the

total amino acid concentrations, as well as the tryptophan levels, were varied. The nicotinamide requirement was tested under these conditions. The results are summarized in table 2. Although the total growth varied in relation to the total amino acid concentrations when these were used in the proportions

TABLE 2

Dose response to nicotinamide in varying concentrations of amino acids

(Values are given in optical density where no growth = zero)

NICOTINAMIDE	SIMULATED CASEIN LEVEL					
	12%	18%	24%	24%	12%	12%
	L-tryptophan (µg/ml)					
	10	15	20	10	10 gelatin (12%)	10 glycine (2%)
µg/ml						
0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.005	0.018	0.002	0.101	0.070	0.001	0.029
0.006	0.039	0.035	0.135	0.115	0.039	0.060
0.007	0.072	0.054	0.162	0.179	0.055	0.093
0.008	0.080	0.090	0.196	0.188	0.086	0.117
0.009	0.100	0.115	0.222	0.211	0.132	0.159
0.010	0.139	0.154	0.247	0.235	0.166	0.181
0.025	0.233	0.299	0.324	0.325	0.236	0.250
0.050	0.251	0.345	0.352	0.358	0.248	0.261
0.100	0.260	0.342	0.354	0.361	0.249	0.259
Amount of nicotinamide for ½ max. growth (µg/ml)	0.0105	0.0105	0.008	0.008	0.009	0.008

characteristic of casein, the requirement for niacin was not appreciably different in any instance. It will be seen from the figures given in table 2 that the addition of 12% gelatin [2] or 2% gylcine neither increased total gowth nor decreased it. This last does not correspond to the results reported by Briggs, Luckey, Tepley, Elvehjem and Hart ('43) and Briggs ('45) on the chick, or with those obtained by Krehl, Sarma and El-

[2] Eastman ash-free.

vehjem ('46), Krehl, Henderson, de la Huerga and Elvehjem ('46) and Schweigert and Pearson ('48) with the rat, where depression of growth resulted which could be alleviated by added niacin or tryptophan.

Dose response experiments were conducted wherein the amount of tryptophan was varied in order to determine the effect of different levels of amino acids on the tryptophan requirement. Some of these results are summarized in table 3.

TABLE 3

Dose response to L tryptophan

(Stimulated casein [minus tryptophan] at the 12% and 18% levels. Both media contain 0.01 µg/ml nicotinamide.)

L TRYPTOPHAN	12% SIMULATED CASEIN LEVEL	18% SIMULATED CASEIN LEVEL
µg/ml		
0	0.000	0.000
2	0.105	0.057
4	0.218	0.230
6	0.260	0.294
8	0.269	0.309
10	0.261	0.342
12	0.255	0.339
14	0.260	0.321
25	0.237	0.332
50	0.209	0.310
Amount of tryptophan for ½ max. growth (µg/ml)	2.5	3.0

The amount of tryptophan required for half-maximum growth increased as the total amino acid levels increased.

Hankes, Henderson, Brickson and Elvehjem ('48) have reported that the addition of threonine or phenylalanine to a low casein diet causes severe growth retardation in rats. Protection is afforded by increased tryptophan levels. Similar experiments were conducted under our conditions and no growth inhibition resulted with increases of either amino acid.

Moreover, the results were the same when the tryptophan level was suboptimum (7.5 μg/ml) or high (27.5 μg/ml) and at low or optimum levels of nicotinamide (0.01 μg/ml and 0.1 μg/ml, respectively).

We have tested the effects of indole acetic acid in media containing varying amounts of tryptophan and nicotinamide. No inhibitory effects were found, as was earlier reported by Kodicek, Carpenter and Harris ('46). This was not unexpected in view of the general failure to confirm the earlier observation (Krehl, Carvalho and Cowgill, '47; Rosen and Perlzweig, '47; Kodicek, Carpenter and Harris, '47). Corn extracts, on the other hand, did produce growth inhibition. A corn extract prepared by us after the method of Woolley ('46) produced approximately 20% growth retardation when used at levels comparable with those used on mice. Essentially the same results were obtained with a corn extract of high "pellagragenic" potency which was kindly supplied to us by Dr. D. W. Woolley. In neither instance, however, was the inhibitory effect of the corn extracts counteracted by the addition of high levels of either nicotinamide or tryptophan or of both together. It appears, therefore, that growth of Tetrahymena is inhibited by some substance in corn, or perhaps by the materials used in the preparation of the extract, but the system or mechanism inhibited is not that concerned with tryptophan or niacin.

DISCUSSION

The results obtained in this study make it perfectly clear that the "germ-free" animal, Tetrahymena, has lost the enzymes necessary for the synthesis of niacin. It would appear, therefore, that this organism has lost an enzymatic mechanism retained by the mammal. When one considers the complications imposed on the investigator of mammalian biochemistry and nutrition by the intestinal microflora, and the possibilities of injected materials diffusing with the intestinal secretions into the alimentary tract where the microflora reside, it is not surprising that differences of opinion exist regarding the actual site of niacin synthesis. To our knowledge

only three "germ-free" animal species have been investigated with the niacin problem in mind. These are the chick embryo: the fruit fly, Drosophila; and the present organism, Tetrahymena.

Snell and Quarles ('41) reported a striking increase in niacin during embryonic growth in the chick. We have confirmed this observation. On the other hand, we were unable to confirm the report of Schweigert, German and Garber ('48) on the effect of injected tryptophan in increasing still further the niacin yield in chick embryos.[3] The fact exists at least that embryonic enzymes are present in the chick for niacin synthesis and the effect of added tryptophan appears unimportant. As yet the niacin precursor in the chick embryo is unknown.

Schultz and Rudkin ('48) report that "germ-free" Drosophila are entirely dependent upon an outside source of niacin and that their requirement is increased by the addition of high levels of tryptophan. Drosophila and Tetrahymena are thus similar with respect to niacin synthesis, although phylogenetically there is perhaps as great a difference between the insects and the protozoa as between the protozoa and the mammals.

The question of whether our results and those of Schultz and Rudkin ('48) merely reflect species variations from the vertebrates so far investigated, or whether animal potentialities for niacin synthesis are in reality more restricted than has sometimes been supposed, must await more widespread experimental use of "germ-free" animals of widely different varieties.[4]

SUMMARY

The ciliated protozoan, *Tetrahymena geleii* W, was grown in a chemically defined medium and tests were conducted to

[3] Unpublished results. Confirmation of Schweigert et al. ('48) has been given by Denton, Kellogg, Rowland and Bird ('49).

[4] Since this paper went to press Henderson and Hankes ('49) and Hundley ('49) have shown that the administration of tryptophan to the rat results in significant increases in niacin synthesis. Since the bacterial flora were largely removed by enterectomy, it appears that there can be little doubt that the site of a large portion of the niacin synthesis from tryptophan is in the mammalian tissue.

determine whether or not there is a relationship between tryptophan and nicotinamide synthesis in this organism. It was found that the level of tryptophan in the medium in no way affected the organism's inabilty to synthesize nicotinamide. Corn extracts, containing the "pellagragenic" agent, produced inhibition but the inhibition was not reversed by tryptophan or nicotinamide or both together. This "germ-free" animal, therefore, is similar to "germ-free" Drosophila in its requirement for exogenous niacin. The implications of these findings in relation to mammals are discussed.

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THE EFFECT OF ORAL ADMINISTRATION OF RUTIN ON BLOOD, LIVER AND ADRENAL ASCORBIC ACID AND ON LIVER AND ADRENAL CHOLESTEROL IN GUINEA PIGS[1]

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ONE FIGURE

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Since the discovery of *citrin* in Szent-Györgyi's laboratory (Armentano et al., '36), numerous authors have postulated a physiological relationship between the capillary permeability factor and vitamin C (Lindheimer et al., '42; Parrot et al., '45; Shanno, '46). In fact, it has been recommended that adequate ascorbic acid intake be ensured in therapeutic treatment with vitamin P preparations (Couch et al., '46). The study presented in this paper was undertaken in an attempt to see whether or not one of the "vitamin P" substances, namely rutin (the rhamnoglucoside of quercetin), might influence ascorbic acid levels in the organism of a species which requires a dietary source of vitamin C.

Previous work in this laboratory (Papageorge and Adair, '47) had failed to give conclusive evidence as to the possibility of a "sparing effect" by rutin on the plasma ascorbic acid of normal human subjects during a period of low C intake. It was decided to investigate this possibility further by use of experimental animals, the ascorbic intake of which could

[1] Supported by a grant-in-aid from Sharp and Dohme, Inc., Glenolden, Pennsylvania. Originally presented before the Division of Biological Chemistry at the New York Meeting of the American Chemical Society, September, 1947.

be controlled better than in volunteer subjects. Guinea pigs were, therefore, placed on adequate and on inadequate diets with respect to vitamin C and the effect of oral administration of rutin on ascorbic acid levels in blood, liver and adrenals was studied. Because of the suggestion that ascorbic acid and cholesterol are both associated with the formation and release of adrenocortical hormones (Sayers et al., '46), liver and adrenals were also analyzed for cholesterol in two of the three experiments. No blood cholesterol determinations were made since we had not observed any effect on serum cholesterol after ingestion of rutin in the preliminary work with human subjects (Papageorge and Adair, '47).

PROCEDURE AND METHODS

Young adult male guinea pigs, weighing initially 335 to 532 gm, were used. Three experiments were carried out, in each of which 8 animals were given rutin and 8 served as controls. The ascorbic acid intake was adequate in the first two experiments and inadequate in the third. Experiments 1 and 3 were terminated on the 22nd day and analyses were made for whole blood, liver and adrenal ascorbic acid, and for liver and adrenal cholesterol. Experiment 2 was designed to reproduce, under better controlled conditions, the significant findings of the first experiment. It was terminated on the 15th day and involved only blood and adrenal ascorbic acid determinations.

The basal diet was a commercial rabbit chow [2] supplemented weekly with oleo percomorphum. Water was given ad libitum. At the outset it was erroneously assumed that the ascorbic acid content of the chow was negligible, so that most of the animals in the first experiment were kept in pairs and food intakes were not recorded. Analysis of the chow not only proved our assumption wrong but showed also that the vitamin C content varied in different batches of chow even when this was exposed to the air as recommended by Sealock and

[2] Purina checkers, "complete ration."

Silberstein ('40) for destruction of ascorbic acid. Therefore, the animals of the second and third experimental groups were kept in individual cages, daily food intakes were recorded, and each batch of chow was analyzed for ascorbic acid. It was thus possible to arrive at a fairly accurate estimate of the total daily intake of ascorbic acid of each animal, as well as to achieve a more nearly uniform food intake than when two or more animals are kept together in the same cage.

Adequate ascorbic acid intake was ensured in the first two experiments by daily oral administration of 1.3 mg of the vitamin [3] per 100 gm body weight (Kuether et al., '44). The average total ascorbic acid intake (chow and supplement) was approximately 1.8 mg per 100 gm body weight per day in experiment 1. The corresponding intake in experiment 2 was calculated as 1.57 mg. No supplementary ascorbic acid was given to the guinea pigs in the third experiment; the daily vitamin C intake of the animals in this group averaged 0.40 gm per 100 gm body weight.

The rutin-fed guinea pigs in all three experiments were given a 20 mg tablet of rutin [4] by mouth every other day. On the day the experiment was terminated rutin was always given, but no food nor supplementary ascorbic acid.

At the end of the experimental period the animals were anaesthetized with nembutal, blood was taken by heart puncture, the organs to be analyzed were removed, and the analytical procedures were carried out. No more than 6 animals were placed on the test diet on a given day, so that the initiation and termination of each of the three experiments were staggered over a period of days in order to make possible completion of the chemical analyses within the required time. Oxalated whole blood was used for ascorbic acid determination. The liver samples and the adrenals were dissected quickly, weighed in 6% trichloraceteic acid on an analytical balance to the nearest 0.1 mg, and ground in the appropriate extracting solution (trichloracetic acid for vitamin C and

[3] Lilly's Cevalin.

[4] Sharp and Dohme, Inc.

acetone-alcohol for cholesterol) with a small tissue grinder. The left adrenal gland was used for ascorbic acid determination and the right for cholesterol except where only the former was determined, in which case both adrenals were used for ascorbic acid. Total ascorbic acid was determined by the technique of Roe and Kuether ('43), and the total cholesterol by Sperry's modification ('45) of the Schoenheimer and Sperry method. The analytical values were calculated as milligrams per 100 ml of whole blood or per 100 gm of fresh tissue.

RESULTS

Figure 1 presents the arithmetic means of the analytical findings. No appreciable difference was found between the rutin-fed animals and their respective controls in blood and liver ascorbic acid levels or in liver and adrenal cholesterol. The rutin-fed guinea pigs in group 1 (1.8 mg ascorbic acid per 100 gm body weight per day) showed a 13.1% greater mean level for adrenal cholesterol than their controls, but the absolute difference was very small and the individual variations were appreciable. The standard deviations from the mean values of adrenal cholesterol for group 1 were relatively great: 4.15 ± 0.71 for the rutin-fed and 3.67 ± 0.76 for the controls. Therefore, the difference of 0.48 mg % is not to be regarded as significant.

The most marked differences between rutin-fed and control animals were found in the adrenal ascorbic acid values, particularly in the first two groups, which were maintained on adequate vitamin C. In both of the adequate-C groups rutin appeared to increase the ascorbic acid concentration in the adrenals. This effect was not evident in the inadequate-C group, in which the rutin-fed animals showed a slightly lower adrenal ascorbic acid level.

Table 1 summarizes the statistical analysis of the data on adrenal ascorbic acid for all three groups. The standard

error of the difference between the means* indicates the same order of doubtful significance for the increase in the group 1 rutin-fed as for the decrease in the group 3 rutin-fed

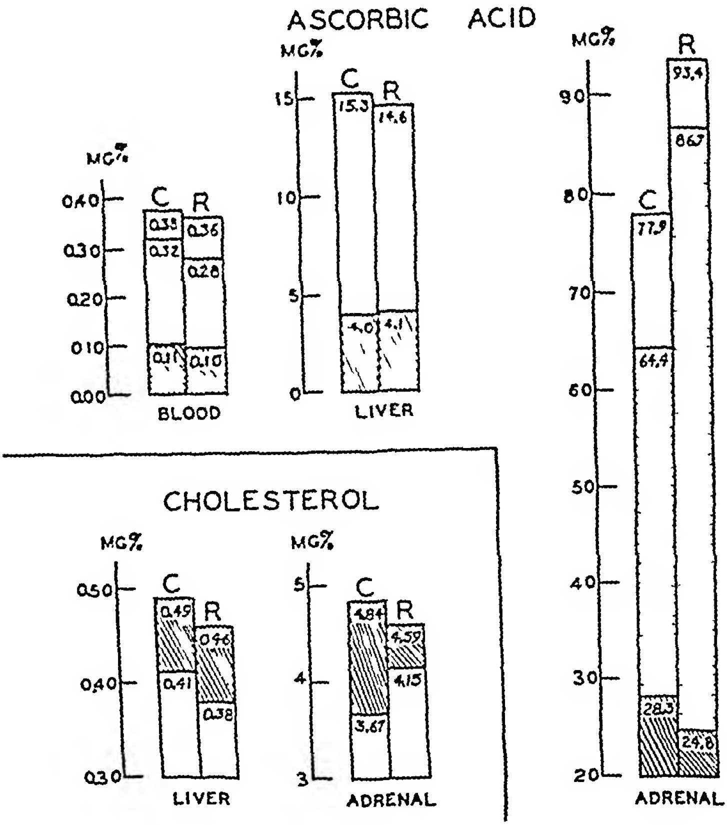

Fig. 1 Ascorbic acid and cholesterol levels in blood and tissues of three groups of guinea pigs maintained on different intakes of ascorbic acid. Mean daily ascorbic acid intake per 100 gm body weight represented as follows: blank areas = 1 8 mg for 21 days (group 1), dotted areas = 1 57 mg for 14 days (group 2); shaded areas = 0 40 mg for 21 days (group 3). C columns = control values, R columns = values for rutin fed animals.

* The standard error of the difference between the means was calculated from the equation for small samples:

$$S_D = \frac{\sqrt{\dfrac{\Sigma(x^2_1) + \Sigma(x^2_2)}{(N_1-1) + (N_2-1)}}}{\sqrt{\dfrac{N_1 N_2}{N_1 + N_2}}}$$

animals. However, the absolute difference between the control and the rutin-fed mean values is relatively small in group 3. Furthermore, the individual variations in food intake and, therefore, in ascorbic acid intake were undoubtedly much greater in the first experiment than in the other two, in which the animals were kept in individual cages. The results of the second experiment supported our conclusion that rutin does increase adrenal ascorbic acid concentration when vita-

TABLE 1

Differences between mean ascorbic acid concentrations of rutin fed and control guinea pigs on adequate and inadequate vitamin C intakes

GROUP	VITAMIN C INTAKE: Mean per day[1]	No. days[2]	ASCORBIC ACID CONCENTRATION IN ADRENALS: Control means	Rutin fed means	Increase on Rutin	Difference between means	S_D[3]
	mg/100 gm		*mg %*	*mg %*	*%*	*mg %*	
1	1.8	21	77.9 ± 16.40[4]	93.4 ± 15.43	19.9	15.5	± 8.25
2	1.57	14	64.4 ± 13.56	86.7 ± 13.15	34.6	22.3	± 6.91
3	0.40	21	28.3 ± 3.76	24.8 ± 3.06	−12.4	3.5	± 1.71

[1] Milligrams of ascorbic acid per 100 gm body weight.
[2] Number of days on indicated intake before analysis.
[3] Standard error of the difference between means.
[4] Standard deviation of mean.

min C intake is adequate. The mean adrenal ascorbic acid of the group 2 rutin-fed guinea pigs was 34.6% greater than that of the corresponding controls. The actual difference between the two means in this experiment was 22.3 mg %, and since this value is more than three times the standard error of the difference between the means, it is statistically significant.

DISCUSSION

The concentrations of blood and liver ascorbic acid in all three groups, both rutin-fed and control animals, are in general agreement with those reported by Kuether, Telford and Roe ('44) for guinea pigs maintained on similar levels of vitamin C but for longer periods of time. The liver con-

centrations found by us are somewhat higher but the difference can hardly be regarded as significant in view of the considerable individual variations encountered in such work. The group 3 animals, maintained on an average daily intake of 0.40 mg of ascorbic acid per 100 gm body weight, had a mean adrenal ascorbic acid value of the same order as that found by Kuether and his co-workers with a corresponding intake of 0.701 mg. However, our controls on adequate C intake had adrenal ascorbic acid levels which compare more closely with those reported by these authors for similar ascorbic acid intakes. This makes more likely our belief that the increase in adrenal ascorbic acid of the rutin-fed animals on adequate ascorbic acid intakes was actually due to the administration of rutin.

Our findings are only in partial agreement with those of Cotereau et al. ('48), who recently reported a "sparing effect" of a "vitamin P" substance on liver, spleen, kidney and adrenal ascorbic acid in the guinea pig. Cotereau and his co-workers found 4 to 8 times more vitamin C in these organs when 3 mg of catechin as well as 10 mg of ascorbic acid were added daily to the basic diet than when ascorbic acid alone was added. Catechin (3, 5, 7, 3′, 4′ pentahydroxy-phenyldihydrochromane) without supplementary vitamin C had no effect on the vitamin C levels of the scorbutic animals nor on their survival time.

Our results neither support nor contradict the suggestion that "vitamin P" is necessary for the absorption and retention of ascorbic acid (Elmby and Warburg, '37; Warter et al., '48); nor do they provide evidence in favor of the theory that one function of "vitamin P" is to lower the need for ascorbic acid (Parrot et al., '45). In the vitamin C-inadequate group we observed no difference in rate of gain of body weight nor in general well-being between the controls and the rutin-fed guinea pigs. Furthermore, if the whole blood level of ascorbic acid reflects its concentration in the tissues and is, therefore, a significant indication of nutritional status with respect to this vitamin (Kuether et al., '44), then rutin

does not appear to lower the need for ascorbic acid, at least under the conditions of our experiments.

The influence of rutin in raising the vitamin C concentration in the adrenal when adequate supplies of the vitamin are available may be the result of its antioxidant effect with respect to ascorbic acid (Rayle and Papageorge, '48) and also toward epinephrine (Lavollay, '41; De Eds, '47), since oxidation products of the hormone markedly accelerate oxidation of ascorbic acid in the presence of cytochrome C (Hermann et al., '46). Kuether and his co-workers ('44) showed that as ascorbic acid intake was increased above the level necessary to prevent any deficiency symptoms (1.23 mg per 100 gm of body weight per day), the concentration in the adrenal rose in greater proportion than in blood, brain muscle, kidney, spleen, heart or liver. It is thus probable that when amounts of ascorbic acid in excess of the minimum necessary to meet metabolic requirements are available, rutin prevents destruction of some of the surplus vitamin through its antioxidant effect and thus permits increased deposition of ascorbic acid in the organ which has the greatest avidity for storing it.

SUMMARY

1. The influence of oral administration of rutin on total ascorbic acid concentrations of whole blood, liver and adrenals, and on total cholesterol of liver and adrenals, was studied in two groups of guinea pigs, one of which was maintained on adequate and the other on inadequate levels of vitamin C intake for three weeks. Sixteen animals were used in each group, of which 8 served as controls and 8 were given 20 mg of rutin every other day throughout the test period. A third group of guinea pigs was maintained on an adequate ascorbic acid intake for only two weeks, and only the blood and adrenal vitamin C concentrations of this group were determined.

2. No significant differences in mean values between the rutin-fed animals and their respective controls were found except in the adrenal ascorbic acid concentrations of the two

groups on adequate vitamin C intake, which were as follows: (1) 77.9 gm % for controls and 93.4 mg % for rutin-fed; (2) 64.4 mg % for controls and 86.7 mg % for rutin-fed.

3. This "sparing effect" of rutin on adrenal ascorbic acid under conditions of adequate vitamin C intake may be due to the antioxidant action of rutin with respect to ascorbic acid and epinephrine, which latter when oxidized contributes to the destruction of vitamin C. Rutin may thus protect some of the surplus ascorbic acid from oxidation and permit increased deposition of the vitamin in the adrenals.

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